TOWARDS GENE THERAPY FOR HAEMOPHILIA USING MUSCLE AS A TARGET:

A STUDY OF VECTOR TRANSDUCTION AND THE IMPLICATIONS OF THE IMMUNE RESPONSE

By

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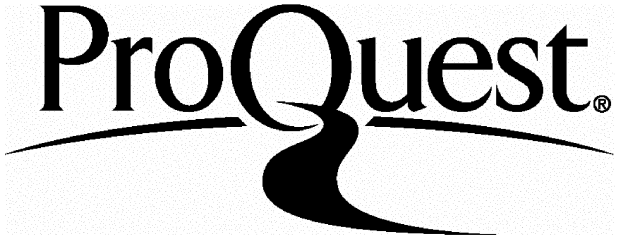

ABSTRACT

A major hope for treatment of haemophilia is via a gene based approach. But with this hope as for any new novel treatment for haemophilia, is the occurrence of an immune response. This thesis attempts to examine the role of the immune response to Gene transfer by a muscle directed approach. It characterises the humoral and cellular immune response to the secretable human transgene product human factor IX in both small and large animal models of haemophilia.

The humoral responses were studied by means of Elisa, Western Blot and Clotting based assays in both normal and haemophilic animal models. The aim of these experiments was to look for the presence of an antibody to human factor IX, determine whether it was inhibitory or not, and look for factors which may influence it's occurrence. Cellular immune responses were analysed histologically, and also by devising an *invitro*, and later an *in vivo* based cytotoxic T cell assay (CTL) assay to look for the presence of specific cellular based responses to the secretable transgene product human factor IX.

The results demonstrated that humoral responses were observed to the transgene product human factor IX, and that the antibody subtype IgG1 was induced, in keeping with a TH2 cellular driven immune response to adeno-associated viral gene transfer. In the case of plasmid and Adenoviral vectors, a more dominant TH1 cellular driven response was observed. In the large animal studies a humoral response was observed, but in this scenario the response was less inhibitory than observed in the mice. The development of a humoral response could be overcome by setting immunomodulatory strategies designed to suppress inhibitor formation. The cellular based responses showed that for Adenoviral based gene transfer, strong Cytotoxic T cell responses were observed, but not for AAV based muscle transduction.

The conclusions from these studies showed that likely immune responses induced were influenced by vector and transgene selection. Other factors such as route of gene transfer administration and underlying mutation status in recipient animals also affected outcome.

Finally it was shown that some of these immune responses could be overcome by the design of immunomodulatory strategies. These findings have significant implications for the design of human clinical studies of gene transfer treatments for haemophilia.

DEDICATION

I wish to dedicate the work in this thesis to Gaynor, Carole and the late Joseph Mitzman.

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ABBREVIATIONS

AAV	adeno-associated virus
Ad	adenovirus
Ag	antigen
Ab	antibody
APC	antigen presenting cell
APTT	activated partial thromboplastin time
bp	base pairs
BU	Bethesda Unit
CD	cluster of differentiation
CD40L	CD40 ligand
CMI	cell mediated immunity
CMV	cytomegalovirus
CsCl	caesium chloride
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte antigen-4
cFIX	canine factor nine
cDNA	complementary DNA
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunoabsorbent assay
E:T	effector: target
FBS	foetal bovine serum
FIX	factor IX
FITC	fluorescence isothiocyanate conjugate
FVIII	factor VIII
HLA	human leukocyte antigen
IgG	immunoglobulin
IL-2	interleukin 2
IL-4	interleukin 4
IL-10	interleukin 10
IM	intramuscular
i.p.	intraperitoneal

ITR	inverted terminal repeat
i.v.	intravenous
kb	kilobase
L	litre
M	molar
mAb	monoclonal antibody
$MgCl_2$	magnesium chloride
Mg_2SO_4	magnesium sulphate
MHC	major histocompatiblity complex
ml	millilitre
mRNA	messenger Ribose nucleic acid
mFIX	murine factor nine
OD	optical density
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT	reverse transcription / room temperature
s.c.	Subcutaneous
SI	stimulation index
TBE	Tris-Borate-EDTA
TCR	T cell receptor
TE	Tris EDTA
Th	T helper cell
µl	micro litre
µM	micromolar
µg	micro gram
WBCT	whole blood clotting time

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CHAPTER 1

INTRODUCTION

1.1 Haemophilia: Historical Perspectives

Haemophilia is an inherited bleeding disorder caused by defective production of either procoagulant protein factor VIII or IX. It is a monogenic disease caused by a molecular defect in either the factor VIII or IX gene on the X chromosome and is, therefore, transmitted in an X linked fashion. The word haemophilia is derived from the Greek word haima, blood, and philein, to love, meaning senseless bleeding. The first use of the word haemophilia was attributed to Schonlein (Virchow, 1854) but the first written use was by Hopf, a student of Schonlein, who gave a dissertation entitled "Die Hamophiliet" at Warzburg in 1828. Clinically the two disorders, deficiency of factor VIII (Haemophilia A) or factor IX (Haemophilia B) are indistinguishable and no differentiation can be made with regard to the bleeding tendency. The first described case of haemophilia as a bleeding disorder was documented over 1700 years ago in the Babylonian Talmud. In early Jewish history the disorder was recognised following circumcision. At this time it was taught from the words of Rabbi Judah the Patriach:

" For it was taught: if she circumcised her child and he died (as a result of bleeding from the operation), and a second one also died, she must not circumcise her third child."

The decree of Rabbi Judah recognised the familial and sex linked nature of the bleeding defect and variations of his edict have been incorporated into Rabbinical law throughout the centuries and repeatedly commented on by others (Maimonnides, 12th century).

The first reported case of haemophilia in the USA was reported in the Massachusetts Gazette of March 22 1791, and described the case of Isaac Zoll who died in Frederick County, Virginia, at the age of 19 from

"A slight cut in one of his feet, with an axe. From the time of his receiving the wound, till he expired, no method could be devised to stop the bleeding."

This obituary describes five brothers who all had died of exsanguination following minor trauma: one received a prick with a thorn, another a scratch with a comb, a third a prick with a needle, a fourth, bruised his cheek against a stove, and a fifth received a cut in one of his thumbs. It is interesting to note that the boy's father, Henry Zoll was twice married and only the children of the first wife were affected by the bleeding tendency.

In 1803, John C. Otto of Philadelphia described a family with a severe bleeding tendency. Otto recorded that only males were subject to a "strange affliction" and that females although exempt, were still capable of transmitting it to their male children. The first pedigree of haemophilia was described by Hay (1813), who followed the disease in the Appleton Swain family through six generations spanning 172 years. This family has since been reinvestigated (McKusick 1962) and been traced over 400 years and 13 generations. The disease in this family originated from a male born in Bristol, UK in 1630, and subsequently went to live in Newbury, Massachusetts in 1639.

The first European series of the disease were case reports by the editor of Sammlung Auserlessener Abhandlungen (1805) and by Consbruch (1810, quoted by Bulloch and Fildes, 1911), followed by careful clinical and post mortem examinations by Blagden (1817) and Wilson (1819). Ward noted the first report of haemophilia being due to a coagulation defect in 1819 but his suggestion was ignored for 80 years. The next publication marked the transition from isolated case reports to describe the disease scientifically. Nasse in 1820 was of the opinion that the blood was of unusual fluidity and that this was due to hyperoxidation. From this study came Nasse's law that stated that haemophilia only occurs in males and is transmitted by unaffected females.

After 1820, the history of haemophilia is almost totally recorded in the German literature between 1830 and 1860. Complete descriptions of the diseases are seen in the German texts of Schonlein, Constatt, Neumann and Fuchs. At this time there was no real clue to the aetiology of the disease but it was thought by Schomnlein to be due to cyanosis and malformation of the heart, whilst others thought it to be due to anomalous gout or scrofula despite the adequate post mortem examinations (Virchow 1854).

In 1893 Wright discovered that the clotting time of the patients blood was prolonged and in 1911 Addis, Minot and Lee (1916) observed that the addition of normal blood could correct the clotting defect in the haemophilic blood. Bulloch and Fildes (1911) surveyed the diseases in 235 patients and their relatives. They re-emphasised the limitation of the disease to males but gave no credence to earlier descriptions of haemophilia in the female, despite early case reports strongly suggesting a bleeding tendency. Indeed the female patient described by Treves in 1886 has been subsequently shown to belong a family afflicted with haemophilia.

In the first few years of the 20th century the diagnosis of haemophilia depended on the clinical and family history plus the finding of prolongation of the whole blood clotting time, (Listen 1839, Wright 1893) with normal levels of prothrombin. Treatment of bleeding episodes upto this point varied from the bizarre to the lethal, despite the demonstration by Lane (1840) of the beneficial effect of blood transfusion. This was rediscovered by Weal (1906) and Minot and Lee in 1916. The use of plasma was then shown by Feisly (1923) to be superior to whole blood, and this treatment then became standard practice for the next 30 years.

Until 1947 haemophilia was considered to be a single disease entity, then Pavlovsky observed that blood from one person with haemophilia could correct the defect in another. This phenomenon was demonstrable both *in vitro* and *in vivo*. Coincident with these investigations, the development of the thromboplastin generation test (Biggs *et al.,* 1952) confirmed that there were indeed two separate groups of patients. This observation was confirmed clinically by the finding of a group of haemophilic patients whose defect could be corrected by the infusion of plasma from a haemophiliac (Aggeler *et al.*, 1952, Biggs *et al.*, 1952). This new disease was called Christmas disease and is associated with a lack of a factor found in the serum called Christmas factor (CF, factor IX or PTC).

As knowledge increased and new clotting tests were devised, it became apparent that there were other bleeding disorders beside haemophilia. Further investigations have shown the need for two additional factors participating in the clotting with tissue thromboplastin, factor VII (De Vries *et al.*, 1949, Landwehr, 1951) and factor X

(Telfer *et al.,* 1956). All these defects present with bleeding which is clinically indistinguishable from that of haemophilia and it is probable that early case records included patients with these defects.

As the pathways of blood coagulation have become more understood, it is apparent that other defects can be found in the laboratory which do not manifest clinically for example, Hageman factor (XII) deficiency (Ratnoff & Colopy, 1955) is not usually associated with bleeding, and deficiency of plasma thromboplastin antecedent (factor XI) (Rosenthal *et al.,* 1953) is associated with bleeding in only about two thirds of cases. Deficiency of fibrinogen may be associated with only a mild bleeding defect.

Classical haemophilia probably exists in about 3-4 per 100,000 of the population in the UK (Biggs & Macfarlane, 1966). A similar incidence is found in Europe (Hardisty & Ingram, 1965) and in the US (Ratnoff, 1960, Lewis *et al.,* 1963). Classic haemophilia is due to mutations in either the factor VIII or factor IX genes, classified as haemophilia A or B respectively. The clinical presentation of haemophilia B is indistinguishable from that of haemophilia A, though the two disorders may be easily separated by routine laboratory testing of FVIII and factor IX activity. The incidence of Haemophilia A is approximately ~1:5000 male births, and for Haemophilia B is ~1:30,000 births. Although the disease has been described in every racial group (Prentice & Ratnoff, 1967), it is said to be uncommon in the American Negro (Lewis *et al.,* 1963), the Japanese (Yoshida, 1961) and the Bantu (Merskey, 1958).

The most famous family with Haemophilia is that of Queen Victoria, who gave birth to eight children, including Leopold Duke of Albany, who had haemophilia. In addition to this, two of her daughters proved to be carriers. It can be truly said that the result of transmission into the Royal Families of Europe had a profound effect on the course of European history. Nowhere is this more poignant than in the case of Alexandra, who became consort of Tsar Nicholas the second of Russia, whose son Alexis, born in 1904, was affected with severe haemophilia. In Victoria's family there was no evidence of any ancestor with haemophilia and it is presumed that the disease was due to a spontaneous mutation within her gene.

Haemophilia and The Royal Houses of Europe

Russia

Queen Victoria can be assumed to have been an obligate carrier, as over 20 relatives in successive generations subsequently inherited the condition. The third child of Queen Victoria, Princess Alice, married the Duke of Hesse at an early age and went onto have seven children, one of whom Frederick, had haemophilia and he died at the age of three years following a fall from a window. Alexandra, the younger daughter of Alice, whilst visiting Russia for the marriage of her elder sister Irene met the Tsarevich Nicholas for the first time as a 12 year old. Five years later they re-met and and were married in 1894 one week after the death of Nicholas's father Alexander III. The St Petersburg hierarchy did not consider the marriage undesirable even though Haemophilia was well recognised amongst Queen Victoria's descendants.

Her youngest son, Leopold, had already died, as had Frederick her grandson. Despite this, the first reported Royal marriage to be declined because of the risk of haemophilia came as late as 1913, when the Queen of Romania decided against an association between that of her eldest son, Crown Prince Ferdinand, and Olga the eldest daughter of Alexandra and Nicholas. This was a little hypocritical since the Queen of Romania was a granddaughter of Queen Victoria and therefore a potential haemophilia carrier. Once Alexandra was received into the Russian Orthodox Church one of her first duties was to maintain the dynasty and produce a male heir.

Although her first children were female, on August 12th 1904 Alexandra gave birth to a male called Alexis. For a month all seemed well with the young prince but it was then noticed that the young Tsarevitch was bleeding excessively and haemophilia was diagnosed, a fact that made Alexandra deeply depressed about transmitting the disease. Nicholas became embattled in the Russian revolution and Alexandra turned to Rasputin for spiritual help in dealing with Alexis's illness. Unfortunately with the defeat in the Russian revolution Nicholas was forced to abdicate with his family including little Alexis. The imperial Royal Family was not granted asylum by any European governments, including Britain and were therefore forced to live in

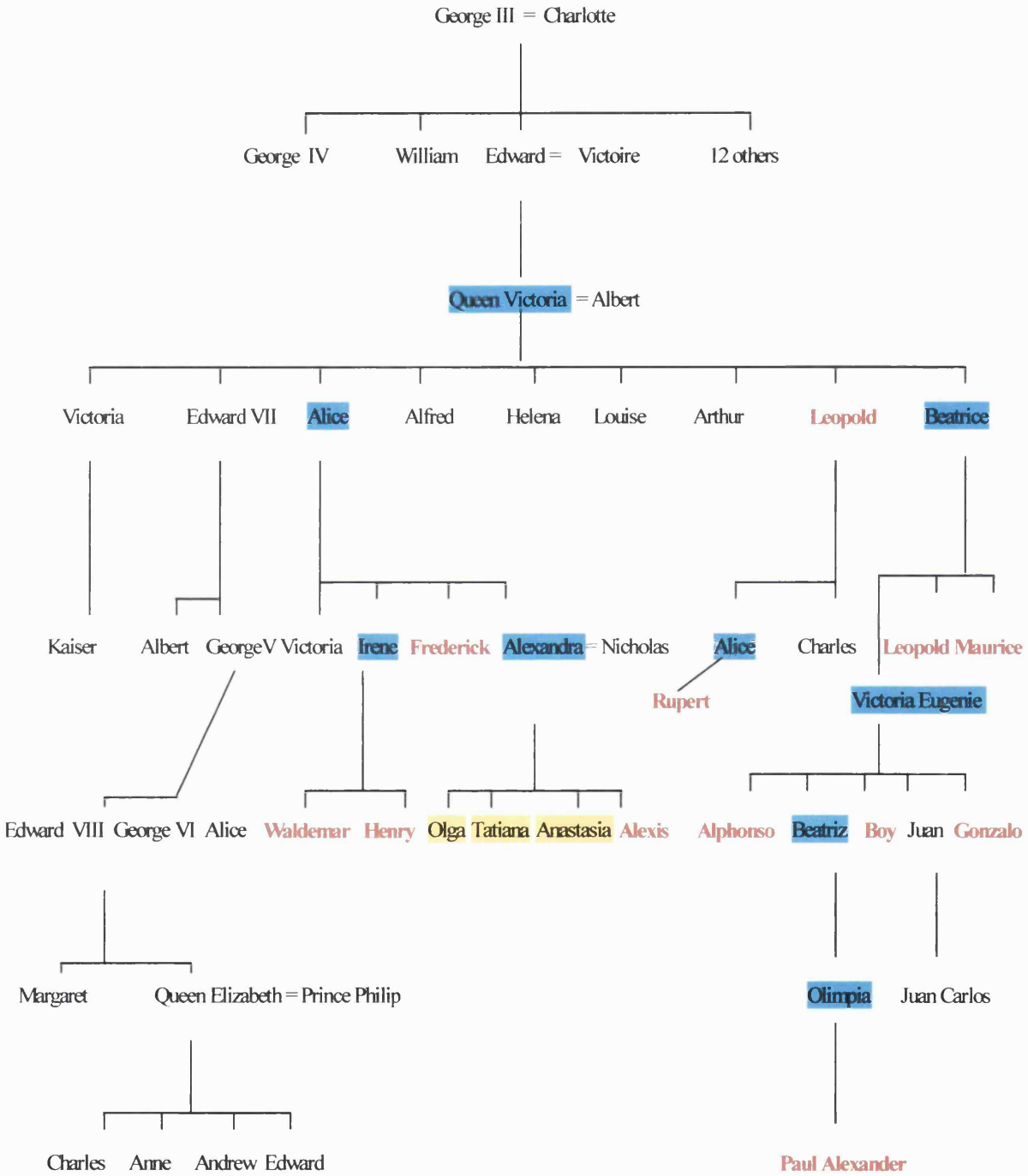
Yekaterinberg until the summer of 1918 when the family were executed by firing squad in the early hours of July 17th.

Spain

Queen Victoria gave birth to nine children over a 17-year period (*see Figure 1*). The youngest, Beatrice, born in 1857 proved to be a carrier for haemophilia and subsequently transmitted the gene to three of her four children. Her daughter Victoria Eugenie, had a significant effect on the political stability of Spain in the early twentieth century. The Spanish King's choice of bride was important and the liberal Alphonso favoured a British queen and he decided to marry Eugenie even though he was armed with the knowledge that she might be a possible carrier. The marriage did not start well and a bomb was thrown at the young couple after their wedding, killing several people. Their first son Alponso had haemophilia and their second son Jaime was congenitally deaf. The third son died young and may well have had haemophilia. The fourth son Juan the father of the present king Juan Carlos was unaffected. There was a lot of anti British feelings in Spain because it was felt that these clinical catastrophes were a direct result of a genetically defective wife defiling Spanish royal blood.

Figure 1 *Royal Family Tree*

Family tree : Queen Victoria's descendants



Key

= Carrier Female

= Possible Carrier Female

Bold = Haemophiliac Male

1.2 Clinical Features

Classical haemophilia is characterised by excessive haemorrhage into various parts of the body. The level of residual factor VIII or IX activity in plasma can accurately predict the severity of bleeding associated with haemophilia A or B. Factor levels of <1u/dl are associated with severe hemorrhagic symptoms, 2-5u/dl levels with moderate haemophilia, and levels of 5-25u/dl with only mild disease.

Roughly 70% of haemophilia patients have severe disease, though this number may be overestimated since patients with severe haemophilia are more likely to seek medical care. Treatment of haemophilia with factor VIII and IX replacement has dramatically improved life expectancy. In the early 1900`s the median life expectancy was only 11.3 years whereas it is currently estimated at between 60-70 years.

Haematomas and haemarthroses are highly characteristic of the disease. The severity of the bleeding tendency varies markedly among families but there is little variation within kindreds. The table below shows the clinical classification based on the severity of the clinical manifestations.

Table 1 Clinical features of Haemophilia

Classification	Factor level (normal range 50-150u/dl)	Clinical features
Severe	**<1u/dl**	Frequent spontaneous haemorrhages and haemarthrosis from early infancy
Moderate	**2-5u/dl**	Haemorrhage secondary to trauma or surgery Occasional spontaneous haemarthroses
Mild	**6-50u/dl**	Haemorrhage secondary to surgery or trauma Rare spontaneous haemorrhage

Severely affected haemophiliac individuals experience spontaneous bleeding. Haemarthroses become frequent at about the time the patient begins to walk and without effective treatment, chronic haemophilic arthropathy occurs by adulthood. Severely affected patients may sustain serious haemorrhages that dissect through tissue planes, ultimately leading to death as a result of compromise of vital organs. Despite the serious haemorrhagic tendency, bleeding episodes are intermittent, with some patients going for weeks or months without haemorrhage. Except for haemorrhage secondary to intracranial bleeding, sudden death due to haemorrhage is uncommon in individuals with severe haemophilia.

Moderately affected patients have occasional haematomas and haemarthroses usually associated with trauma. These patients may have factor activity of 2-5u/dl, just barely enough to limit the frequency of bleeding episodes. Although chronic haemophilic arthropathy may occur in moderately affected patients, it is usually less disabling than that of severely affected patients.

Mildly affected patients have infrequent bleeding episodes and the disease may go undiagnosed for many years, and be discovered because of excessive postoperative bleeding, following trauma, or after severe contact sports.

1.3 Coagulation Cascade

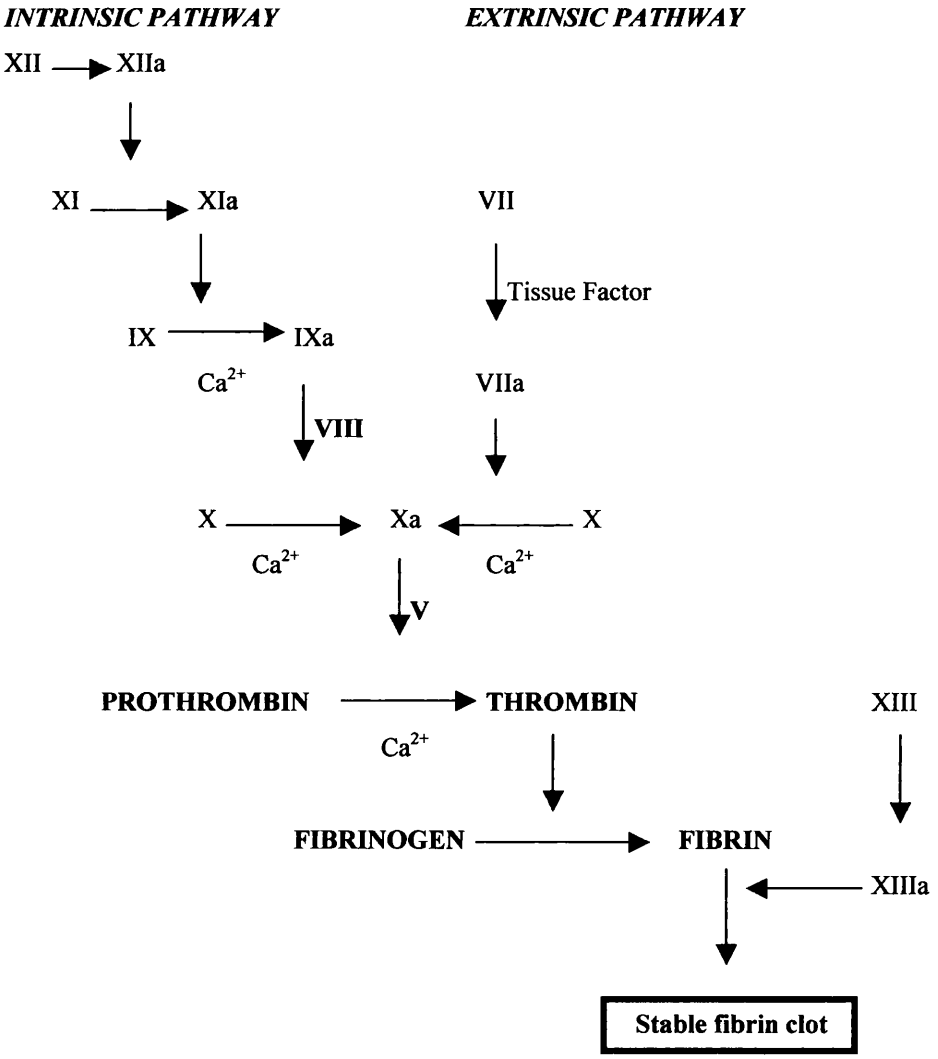
Blood coagulation is a host defence mechanism that protects the integrity of the vascular system after injury. After tissue injury the integrity of the vascular system is maintained by blood coagulation (haemostasis). Haemostasis involves the participation of coagulation proteins, platelets, vascular endothelium, and leucocytes, which lead to formation of platelet plug, fibrin based clot and deposition of white cells at the point of injury and activation of inflammatory and repair processes.

1.3.1 Classical coagulation

Classically the sequential activation of coagulation proteins that leads to the generation of thrombin has been described as two distinct pathways. The classic waterfall hypothesis for coagulation proposes the intrinsic and extrinsic pathways (see

figure 2). Both these systems are routinely assessed in the laboratory. The activated partial thromplastin time measures the activity of the intrinsic system and is based on the contact activation of factor XII, whilst the extrinsic system function is measured by the prothrombin time. This scheme of the division into two pathways remains invaluable for the understanding of clot formation *in vitro*, in monitoring of clinical bleeding problems and monitoring anticoagulant therapy.

Figure 2 *Classical Coagulation Pathway*



Although the classical waterfall hypothesis is useful for measuring *in vitro* assessment of coagulation it fails to represent what happens *in vivo*. This may be pointed out by the following; patients with an inherited deficiency of factor XII, prekallikrien or high molecular kinogen manifest with no clinical bleeding problems, but they have prolonged activated partial thromboplastin times. These clinical observations

demonstrate that these proteins are probably not important components of blood coagulation and should not therefore be considered an important component of *in vivo* blood coagulation.

Factor VII – tissue factor is known to activate not only factor X but also factor XI. In the classic waterfall hypothesis this activation is not required. Tissue factor is a natural constituent of many non-vascular cells. Tissue factor on such cells is able to initiate blood coagulation. These points suggest a central role for the tissue factor-factor VII complex. Further evidence for a revised process of coagulation has been aided by the identification of an endogenous inhibitor of tissue factor induced coagulation (tissue factor pathway inhibitor).

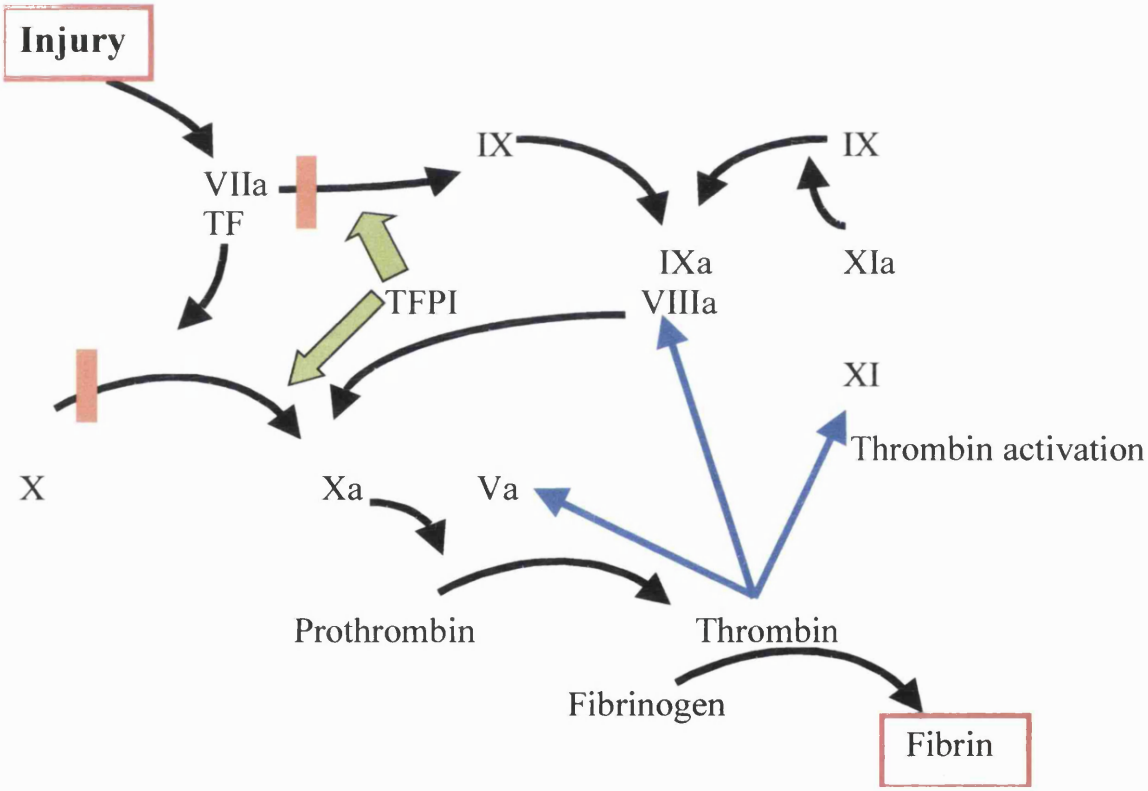
1.3.2 Revised Hypothesis of Coagulation

The findings described above concerning the direct activation of factor IX by tissue factor-factor VII have lead to a revision of the coagulation cascade, with tissue factor-factor VII and factor X central to the model. The revised cascade is believed to more accurately represent what happens *in vivo*. Initiation of coagulation is brought about by exposure of blood to tissue factor, produced constitutively by cells beneath the endothelium.

When tissue factor comes into contact with circulating factor VIIa, it forms a complex in the presence of calcium ions. Factor VII is converted to its active VIIa serine protease by minor proteolysis (possible from trace factor Xa or other protease). The now active factor VIIa-tissue factor complex, then converts factor IX to IXa and factor X to Xa. The newly generated IXa forms a complex with VIIIa (activated by traces of thrombin generated slowly by Xa) in the presence of calcium and membrane phospholipid and subsequently also activates factor X to Xa. The complex is called "tenase". Factor Xa binds to Va (activated by thrombin), which with calcium and phospholipid is called prothrombinase, the complex that rapidly converts prothrombin to thrombin.

The initial TF-VIIa complex is enhanced by feedback activation of VII by factors Xa and IXa. However the complex is quickly inhibited by TPFI (tissue factor pathway inhibitor), a high affinity, low abundance protein found in plasma and on vascular cells. By this time the thrombin that has been produced activates factor XI as well factors V and VIII and therefore augments the formation of tenase and ultimately the production of more thrombin. Thus both the initial stage (TF-VIIa) and subsequent enhancement by intrinsic tenase are necessary to achieve optimal activation of the system.

Figure 3 *Revised Hypothesis of Blood coagulation*



The revised hypothesis differs from the classical one in three main respects. It integrates all coagulation factors known to be involved in blood coagulation into a single pathway. It also assumes that contact activation is not required *in vivo*. Thirdly it also does not assume that initial generation of factor Xa and thrombin is the end of the haemostatic process.

The revised hypothesis assumes that following initial generation, the haemostatic response must be reinforced and or consolidated by a further progressive generation of factor Xa and thrombin. The revised hypothesis (Rapaport & Rao, 1995) helps explain the bleeding seen in haemophilia, both spontaneous (intrinsic) and traumatic (extrinsic). From the revised hypothesis an absence of either factor VIII or IX will lead to a failure of amplification and consolidating generation of factor Xa, and lead to continued bleeding.

1.3.3 The role of factor IX

Factor IX and VIII are both critical components of the enzyme complex that catalyses the cleavage of factor X by the IXa-VIIIa complex to activated factor X (Xa). In the reaction factor IXa is the enzyme, and the factor VIIIa the cofactor that catalyses the conversion of factor X to factor Xa. The reaction also requires phospholipid and calcium. Activation of factor X involves the cleavage of Arg^{194}-ile^{195} within the heavy chain. The cofactor probably serves to orientate the enzyme and substrate (Brandstetter *et al.*, 1995). Thus, under physiological conditions, the reaction does not proceed in the absence of functional factor VIIIa

1.4 Molecular Genetics of Haemophilia

The cDNAs for factor IX and VIII were cloned in 1982 (Kurachi *et al.*, 1992) and 1984 (Gitschier *et al.*, 1985). The availability of this data has paved the way for rapid advances in the molecular characterisation of the defects responsible for the haemophilias (Giannelli & Green, 1996), the production of recombinant clotting factor concentrates for therapeutic use (White & Roberts, 1996), the generation of knockout animals to use as disease models of haemophilia (vide *infra*), and the production of wild type and mutant proteins for analysis of structure function analysis of proteins (Toole *et al.*, 1986, Yamaguchi *et al.*, 1993).

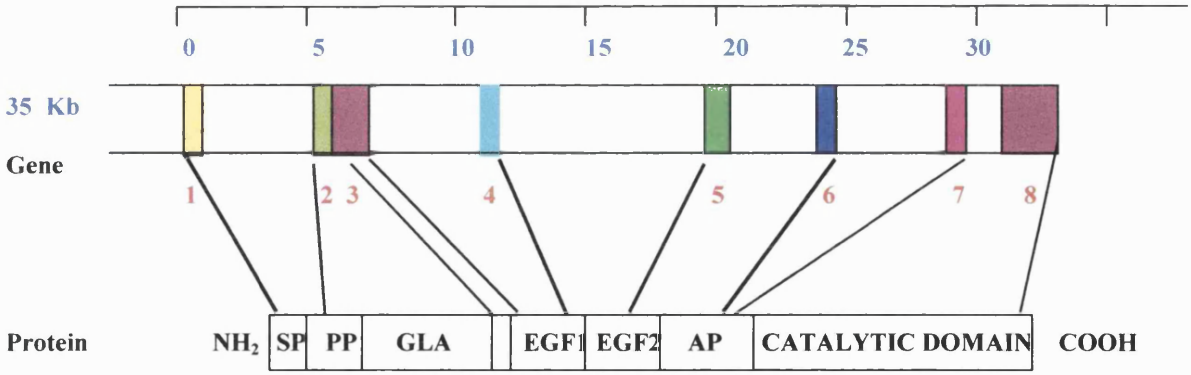
1.4.1 Factor IX gene

By contrast with the factor VIII gene, the factor IX gene is much smaller. The factor IX gene occupies 33.5 Kb of the long arm of chromosome X and is situated at

position 27.1. The gene itself is composed of 8 exons and 7 introns. The structure of the vitamin K dependent clotting factors is strikingly similar at the protein level and at the level of gene organisation. Each of the 8 exons corresponds to a domain of the protein. Exon 1 encodes the signal peptide, exon 2 the propeptide and the amino terminal glutamic rich domain, the Gla domain, exon 3 an aromatic acid rich stack, exons 4 and 5 two epidermal growth factor like domains, exon 6 the activation peptide, exons 7 and 8 the catalytic domain.

The product of the factor IX gene is a polypeptide of 415 amino acids (AA) and proceeded by a pre pro signal peptide. The circulating factor IX consists of the GLA domain and two-epidermal growth factor like domains separated from the serine protease domain by an activation region. The polypeptide undergoes cleavage of the pre and pro segment of the signal peptide prior to secretion as well post translational modifications that include carboxylation of the first 12 glutamic acid residues, O linked glycosylation at Ser 53, Ser 61, Thr 159, and Thr 169 (Agarwal *et al.*, 1994), N linked glycosylation at Asn 157 and Asn 167, and B hydroxylation of Asp 164. Activation of factor IX entails excision of the peptide containing residues 146-180.

Figure 4 *Factor IX Gene and Protein structure*

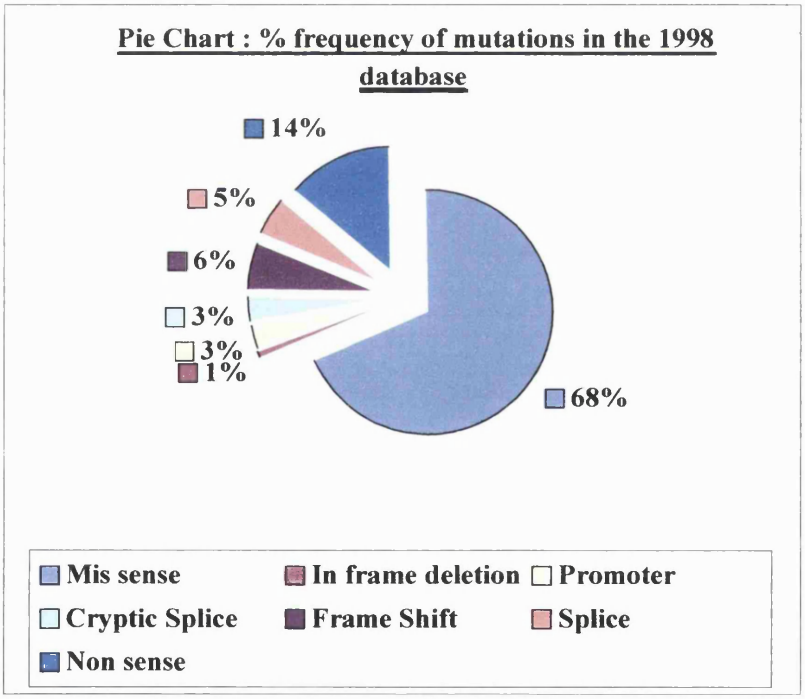


1.4.2 Factor IX Gene Mutations

Progress in molecular biology and the use of polymerase chain reaction has yielded a significant expansion in the detailed analysis of haemophilia B mutations. With the advent of PCR and rapid screening procedures based on PCR (e.g. single stranded conformation polymorphism) the pace of detection has increased dramatically. To

utilise these data effectively, a database in a concise format is now published yearly in Nucleic Acids Research (Giannelli *et al.*, 1998) and available on the world-wide web (http://www.kcl.ac.uk/ip/petergreen/haemBdatabase.html). In the last published edition (7th edition) of the database there are 1713 patient entries. The majority are accounted for by point mutations, 132 have short (<32nt) deletions or additions or both which are made up from 99 deletions, 25 additions and eight examples involving both additions and deletions. There are also 21 double mutations and one triple mutation. It should be noted that the database excludes patients with partial or complete gene deletions or more complex rearrangements. A pie chart showing the relative frequency of each mutation is shown below.

Figure 5 *% Mutations in 1998 Haemophilia B database*



Gross Gene Defects

Gross deletions are the main cause of gross gene defect in haemophilia B. Such mutations only account for 2-3 % of haemophilia B cases, and are usually associated with severe disease

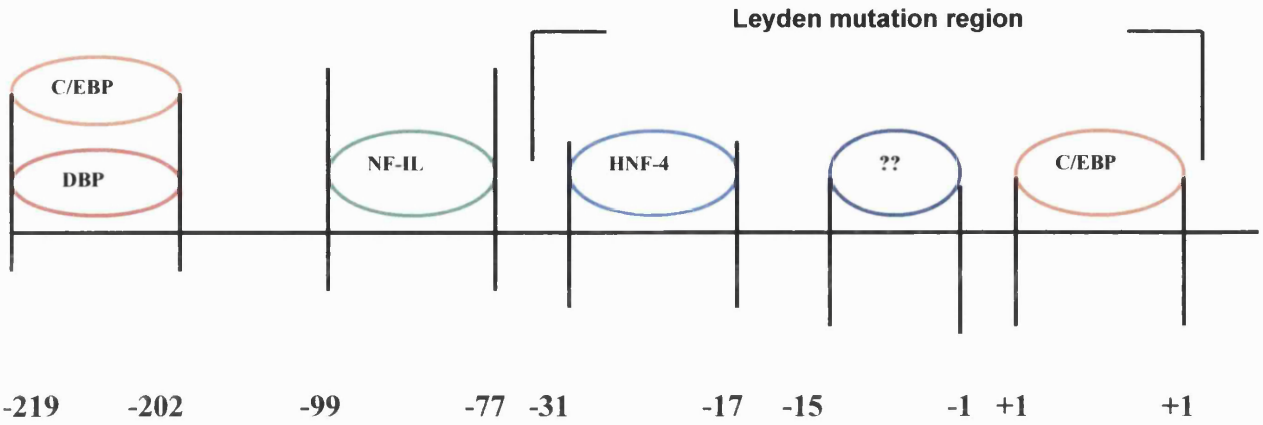
Small gene defects

There have now been reports of mutations in all regions of the factor IX gene. Many of the mutations have been noted to occur at CG doublets, resulting in a C→ T or G→ A transition (Koeberl *et al.*, 1989). This occurs because cytosines are often methlylated, which makes the protein unstable and susceptible to deamination. With deamination the methylated cytosine is converted to thymidine and since thymidine is a normal nucleotide it is not repaired and a mutation results. CG doublets appear to be hotspots for mutations and this may explain how the high number of sporadic mutation account for about one third of cases of haemophilia B. Some of the more frequent mutations do not involve CG doublets but are due to founder effect (Ketterling *et al.*, 1991a, Ketterling *et al.*, 1991b).

Promoter effect and mutations

Promoters are regions of the gene, which bind RNA polymerase and are therefore near the start site of transcription. Regulation of transcription occurs in the promoter region. Regulation can be mediated by *CIS* acting agents, which are endogenous DNA sequences, or by transacting elements. Transacting elements are exogenous factors, which bind to the promoter region and regulate transcription (Gilland & Tijian, 1992). These affect the transcription of the factor IX gene and have helped identify important elements of the factor IX gene promoter. These are nt+1 to +18, -14 to –1, -27 to –16, and –32 to –22. The first, binds members of the C/EBP family of transcription factors, the second proteins are not yet well characterised and the third resembles an androgen response element (Crossley *et al.*, 1992).

Figure 6 *Factor IX Promoter Mutations*



The region in which results in the Leyden mutation is shown. The region binds trans acting elements, including the hepatic nuclear factor-4 (HNF-4), the CCAAT/enhancer binding protein (C/EBP), and an unknown protein (??). DBP= D site binding protein; NF-IL= nuclear factor 1-liver. (Updated and modified from High K.A and Roberts H.R, Molecular Basis of Thrombosis and Haemostasis 1995).

Substitutions in the promoter region of factor IX have classically resulted in the factor Leyden phenotype. This phenotype was first described in 1970 by Veltkamp and colleagues (Veltkamp *et al.*, 1970). Most patients with the phenotype have a severe haemorrhagic disorder and lack factor IX antigen and activity at birth. At puberty, however, there is gradual increase in factor IX levels at a rate of 4-5 % per year until the patient reaches about age 20 when the increase in circulating factor IX alleviates the haemophilic condition.

1.4.3 Factor VIII gene

The factor VIII gene spans 186Kb across 26 exons near the tip of the X chromosome (Xq28). The factor VIII RNA is approximately 9Kb in length and encodes an approximately 300kd protein. The factor VIII protein circulates in the plasma at very low levels (approximately 100mg/ml) where it serves as a critical cofactor for factor IX in the proteolytic activation of factor X to its active form (Xa). Factor VIII is an intrinsically unstable protein, requiring strong electrostatic interaction with Von Willebrand factor (VWF) for stability in plasma. The two proteins circulate together as a tight complex in plasma. The extreme instability of factor VIII in the absence of

VWF accounts for the markedly reduced factor VIII levels observed in severe Von Willebrand disease (VWD) and in patients with variant type 2N VWD.

The Haldane hypothesis predicts that one third of all patients with an X linked lethal disorder should represent new mutations. This results from the fact that one third of all X-chromosomes reside in males and two thirds in females. The frequency of haemophilia A is generally estimated at between 1:10000 and 1:5000 males. Based on this gene frequency, approximately 1:25,000,000 to 1:100,000,000 females would be expected to be homozygous for a factor VIII deficient allele. Consistent with these predictions, classic haemophilia A in females has only very rarely been reported and other possible explanations should be considered whenever a female with this phenotype is encountered. Extreme skewing of X chromosome inactivation can result in unusually low factor VIII levels in female haemophilia carriers. Though this can produce reductions of factor VIII levels into the mild or even moderate haemophilia range, to reach levels <2u/dl is rare and therefore associated severe bleeding is extremely unlikely.

The exception are the rare individuals who carry a haemophilia A mutation associated with an X chromosome: autosome translocation or other cytogenetic abnormality, which may result in exclusive inactivation of the normal X chromosome. If the rearranged chromosome carries a haemophilia A mutation, this female could present with classic haemophilia. The diagnosis of homozygous type 2N VWD should also be considered in any female with very low factor VIII levels.

1.4.4 Intron 22 factor VIII gene inversion

A unique rearrangement within the factor VII gene has been identified as a common recurrent mechanism for haemophilia A (Naylor *et al.*, 1993). This rearrangement is an inversion within the factor VIII gene that is the result of unequal crossing over between a duplicated sequence within intron 22, termed gene A, and two highly homologous blocks of sequence located downstream of the factor VIII gene toward the telomere of the X chromosome arm. The rearranged gene is disrupted at intron 22. The resulting truncated protein product is presumed unstable and does not accumulate to significant levels. Thus the common factor VIII gene inversion is associated with

undetectable levels of factor VIII (<1u/dl), and a typical severe haemophilia phenotype. It is thought that the specific configuration of the factor VIII gene and the repeated gene A elements, as well as their location on the on arm of the X chromosome, make this region particularly prone to rearrangement, leading to high frequency of this recurrent mutational event, which is now known to account for approximately 45% of all patients with severe haemophilia A.

Another interesting observation of the recurrent factor VIII intron inversion is the fact that the mutation always occurs during a male meiosis. This is presumably due to the large region of non-homology between the X and Y chromosome during meiotic pairing, favouring a misalignment between the gene repeated sequences and an illegitimate recombinant event, resulting in the gene inversion. The presence of a second X chromosome with full pairing in this region inhibits this event in a female meiosis, and this observation has important clinical implications. For a patient with an apparently new mutation in whom a gene inversion is identified, the mother can be generally assumed to be a carrier, with the recombination event often identified in the maternal grandfathers' allele.

1.4.5 Other factor VIII gene mutations

The remaining 55% of severe haemophilia A patients who do not carry the intron 22 inversion can generally be shown to have a more conventional defect in the factor VIII gene. Approximately 5% of patients have deletions removing varying sized segments of the factor VIII gene. The remaining defects comprise a small group of gene insertions/deletions and a much larger group (roughly 50%) of specific point mutations in exons or at splice junctions within the factor VIII gene. Additionally nearly all patients with mild or moderate haemophilia A (in which some residual level of factor VIII activity can be demonstrated) can be shown to have a point mutation within the factor VIII coding sequence, resulting in a single amino acid substitution.

Depending on the nature of the amino acid substitution, a range of residual factor VIII activities are observed accounting for the variation in severity among mild and moderate patients.

Mutations which result from C (cytosine) to T (thymidine) transitions at the CpG dinucleotides are very common, accounting for approximately one quarter of single based substitutions. This hypermutability appears to result from the more frequent methylation of Cs at CpG dinucleotides. Methylcytosine can spontaneously deaminate to Uracil, which will be converted by the cells DNA repair machinery to thymidine. A database of factor VIII gene mutations (Tuddenham *et al.*, 1994) has been maintained for haemophilia A that catalogues analysis from over 1000 DNA samples. Most recently in May 1999, 309 single based substitutions had been described with 264 (85%) leading to a single amino acid substitutions (mis-sense mutations) and 45 (15%) to premature stop codons (non sense mutations). An additional 38 mutations may lead to aberrant RNA splicing. Ninety two gene deletions have been reported ranging from 1Kb to 210Kb in size and overall accounting for about 5% of haemophilic patients.

1.5 Treatment of Haemophilia

Major progress in the treatment of haemophilia really took place in the last third of the 20th century. The development and availability of clotting factor concentrates (including Von Willebrand factor, factors VIII, IX, VII, XI, and XIII and fibrinogen) has revolutionised treatment. Increasingly during this period there was also widespread uptake of home treatment, which enabled haemophiliac individuals to lead more fully active normal lives with the freedom to integrate fully into society. The mainstay of treatment was the use of concentrates to simply replace the missing clotting factors and thereby lead to successful secondary prevention of a hitherto severe and disabling chronic disease. The term subsequently used for this form of therapy was "replacement therapy". Replacement therapy with factor concentrates (factor VIII or IX) is the most important part of haemophilia care. The development of plasma and then recombinant clotting factor concentrates has revolutionised haemophilia care. The problem of viral transmission by plasma-derived concentrates has been overcome by the use of plasma donor screening, virucidal killing methods and more recently the use of recombinant clotting factor concentrates.

1.5.1 Replacement Therapy

The cornerstone of haemophilia treatment is replacement of the deficient clotting factor using a purified concentrate of factor VIII or IX preferably given as home therapy, by the patient himself or by a parent in the case of small children. Treatment may be given either on demand or as prophylaxis. On demand therapy is preferably given on the first evidence of a bleed. Home therapy ensures that the patient receives the treatment early in the course of a bleed.

Definition of Prophylaxis

Prophylaxis may be defined as intravenous injection of clotting factor concentrate in anticipation of and to prevent bleeding. Prophylaxis may be given as a single dose procedure that is given prior to an event that may either result in bleeding or be disrupted by it (i.e. surgery), as a short term procedure to counter a temporary increase in the frequency of bleeding, or a long term (primary, permanent, continuous) procedure in order to prevent haemarthrosis and the development of arthropathy.
The term secondary prophylaxis has been used to denote prophylaxis started after the onset of serial bleeds when arthropathy is already manifest. Long-term prophylaxis is usually meant when the term prophylaxis is used.

Long Term prophylaxis

During the 1960`s and 1970`s a number of studies were published describing numerous attempts at the prevention of bleeding. Although some of the studies had obvious deficiencies such as lack of controls, low doses of concentrates, long treatment intervals, they showed that prophylactic treatment could reduce bleeding frequency (Kasper *et al.*, 1970; Ramsay & Parker, 1973).

Value of prophylaxis in children

One of the driving forces for prophylaxis in haemophilia is the prevention of haemophilic arthropathy. The most extensive experience of long-term prophylaxis was that of the series reviewed by Nilsson et al in 1992 (Nilsson, 1992). Prophylactic

treatment of haemophilia A was started on a small scale in Sweden in 1958 and for haemophilia B in the late 1960`s. The rationale for prophylactic treatment was the observation that chronic arthropathy was less frequent and less severe in moderate haemophilia (i.e. factor VIII or IX concentrates 1-4u/dl) than in severe (i.e. factor VIII/IX level <1u/dl) haemophilia.

In the 25-year survey carried out by Nilsson et al, a series of 65 patients with severe haemophilia A were divided into three age groups. In the group of oldest patients (n=25, born 1958-72, age 21-35 at follow up) prophylactic treatment was started at various times between 3 and 13 years of age, and several patients already had arthropathy prior to the start of treatment. Initially they were given considerably less annual dosages than those in use today and the infusions were sometimes given only once a week. The treatment was successfully optimised and the mean annual incidence of joint bleeds being 5 (range 0.5-16), the mean orthopaedic joint score 5.1 (range 0-15) and the mean radiological score 18 (range 0-41).

In the next group which was composed of 19 boys born 1971-1981, (aged 13-20 at follow up) prophylaxis started at 1-4 years of age, usually being given twice weekly in dosages (800-2500 IU/kg/year) somewhat higher than those used in the previous group and successively increased to an average of 5400 IU/kg/year. The mean annual incidence of joint bleeds was reduced to 2.6 (range 0.2-17), the mean orthopaedic joint score was 1.2 9 range 0-7 and the mean radiological score 4.8 (0-22). Of the 19 boys, 14 had orthopaedic scores of zero and 11 had radiological scores of zero i.e. this group had only minor non-disabling arthropathy.

The youngest group of 21 boys, born between the years 1981-90, aged 3-12 at follow up started prophylactic treatment at the age of 1 or 2.The annual dosages ranged from 400 to 9000 IU factor VIII per kg body weight. There were almost no bleeding episodes in these cases. The orthopaedic and radiological scores were zero in all 21 patients. In this youngest group it was usually possible to maintain the factor VIII or IX concentrations above 1u/dl between infusions.

Although a weakness of this study was the lack of a control, the findings clearly demonstrated that more intensive prophylactic treatment received by the youngest

children designed to maintain plasma factor VIII or IX levels above 1u/dl, yielded better results than earlier regimens with lower dosages.

Recommendations were then made by the international and national authorities at a joint WHO-WFH meeting in Geneva in 1994 (Lusher 1995). Since the main goal is to prevent joint bleeding and its sequelae, prophylaxis should be considered optimal management for persons with severe haemophilia A and B (baseline factor VIII/IX levels <2u/dl). Treatment should be started at the age of 1-2 years and be continued indefinitely. These recommendations have now been taken up by the United States National Haemophilia Foundation, Canadian Haemophilia Society and the United Kingdom Haemophilia Centre Directors Organisation, in addition to prophylaxis being the general policy in Sweden, Germany and a number of other European states. Where prophylaxis is not feasible or appropriate, on demand therapy should be given.

1.6 Complications of Treatment

1.6.1 Inhibitor Formation

The development of an inhibitor, an alloantibody that blocks procoagulant function, is one of the most serious complications of haemophilia A or of haemophilia B treatment. Currently the incidence of inhibitor formation in the conventional protein based approach for haemophilia is roughly 20% for Haemophilia A (Briet *et al.*, 1994) and 4% for haemophilia B (Briet *et al.*, 1984).

The clinical impact of inhibitor formation has been recognised for over half a century, and only very recently have studies begun to clarify our understanding of inhibitor development. Recent studies have focussed on four main areas, a better understanding of the incidence of inhibitors following coagulation factor treatment, the identification of patients at the highest risk of inhibitor formation, the characterisation of the antibodies, and the development of better therapies.

Clinical features of inhibitor formation

The typical bleeding frequency or location of bleeding does not change in haemophiliac patients when an inhibitor develops, and joint haemorrhages continue to be a major problem. The development of an inhibitor should be suspected if a patient does not respond appropriately to treatment.

Tests may reveal the rapid clotting factor disappearance from plasma by *in vitro* assays. The inhibitor level does not correlate well with the frequency of bleeding, but it does limit the kind of treatment, that is possible.

Inhibitor incidence

Clinically the development of inhibitors has significant implications for the patients, with resulting decreased life expectancy and increased morbidity. Inhibitors have not been recognised in haemophilia A patients before exposure to factor VIII. Although antibodies may be detected after as few as five treatments, the likelihood of

developing an inhibitor appears to be related to the number of exposures (Ehrenforth *et al.*, 1992). Thus during the first 5-10 years of life, the incidence of inhibitors in haemophilia increases with the number of factor VIII infusions, and new inhibitors are rarely detected after 90 –100 exposure days. The frequency of patient assessment is a key issue in evaluating inhibitor incidence. For example, several studies have detected low titre inhibitors that were not suspected on clinical grounds and that were only identified because of routine assessment every 3-6 months.

It is controversial whether the likelihood of inhibitor formation is related to the purity of the administered factor VIII or to its production process. The issue was originally raised when more highly purified factor VIII concentrates were introduced, and it has been of special interest since the introduction of recombinant concentrates, since there exists subtle differences in the processing of these products, which may alter immunogenicity.

There are some differences in the in the carbohydrate composition of recombinant factor VIII when compared to that purified from pooled normal plasma. However it is very uncommon for previously heavily transfused patients to develop an inhibitor when treatment was changed to monoclonal antibody purified or recombinant factor VIII (Schwartz *et al.*, 1990; Addiego *et al.*, 1993; Bray *et al.*, 1993).

As inhibitors develop early on in life most efforts to determine inhibitor incidence have looked at the response to factor VIII in previously untreated patients (PUPS). The interpretation of inhibitor incidence has been difficult for these new products since there are no previous prospective studies. Until the late 1980`s, inhibitor assessments were retrospective, and were based on the development of resistance to factor VIII treatment – with *in vitro* confirmation that a factor VIII inhibitor was responsible.

Unfortunately, the sequential introductions of intermediate purity concentrates, the monoclonal antibody purified factor FVIII concentrates and recombinant factor VIII were not systematically evaluated for possible product related changes in inhibitor risk. The various retrospective analyses have produced contrasting results. For example while inhibitor development was infrequent in patients treated with

cryoprecipitate in Belgium (7%) (Peerlinck *et al.,* 1993b), patients treated in the US had a much higher incidence (35% Strauss et al., 1969). In the case of intermediate purity products, inhibitor incidence has been reported to vary from less than 20% to almost 30% (de Biasi *et al.*, 1994). In a paper by Yee (Yee *et al.*, 1997), an absence of inhibitors was demonstrated in previously untreated patients with severe haemophilia A after exposure to a single intermediate purity factor VIII product.

Briet (1994) summarised the key data for 8 of these studies, limiting the comparison to those patients at highest risk (severe haemophilia A) who developed a high response inhibitor. The cumulative incidence of high response inhibitors reached a plateau at 20 % for this group of 451 patients after 18 years of follow up (Briet *et al.*, 1994). However, the individual studies had cumulative incidence values from 2 to 46%, demonstrating the hazard of drawing conclusions from relatively small groups of patients.

Recent reports from the recombinant factor VIII trials provided the first detailed prospective characterisation of inhibitor development in previously untreated patients with severe haemophilia A. This multi institution study reported data from patients with severe haemophilia A, who had no prior exposure to any blood products and who were tested for inhibitor development every three months. At the times of publication, 14 (29%) of the 49 patients treated with *Kogenate* had developed an inhibitor (Lusher *et al.*, 1993), as had 17 (24 %) of the 73 patients treated with *Recombinate* (Bray *et al.*, 1994).

Laboratory diagnosis of Inhibitors

The slow kinetics of factor VIII inhibition must be kept in mind when evaluating patients suspected of having an inhibitor. Inhibitor screening is usually performed by incubating equal volumes of patient and normal plasma for one hour before the APPT measurement. If an inhibitory antibody is present, then factor VIII will be inactivated and the APTT prolonged. This assay is not sufficiently sensitive to detect some weak inhibitors.

Quantitative inhibitor assays are an important guide to therapy and to detect changes in the inhibitor level. They are based on the measurement of the amount of factor VIII inactivated when the patient plasma is mixed with normal plasma. Most laboratories use the Bethesda method. In this assay patient plasma, undiluted or diluted with imidizole buffer, is added to an equal volume of pooled normal plasma. After a 2-hour incubation at 37°C, the factor VIII value for the mixture is compared to that of a control in which buffer replaced the patient plasma. One Bethesda unit is defined as the amount of antibody that causes a reduction in residual factor VIII to 50% of the control value (Kasper *et al.*, 1975). The patients' levels are calculated by extrapolation from the plasma dilution that yields a 50% residual factor VIII level. Less commonly used variants include the New Oxford assay, where a factor VIII concentrate is the source of factor VIII, and the incubation is carried out for 4 hours at 37°C (Austen *et al.*, 1982). One Bethseda unit is equivalent of 1.21 New Oxford units (Austen *et al.*, 1982)

1.6.2 Management of Inhibitors

As more inhibitors are seen in haemophilia A, most studies addressing the management of inhibitor formation have centred on haemophilia A. Most of the data reported in this section, therefore, discusses treatment for haemophilia A.

There are two issues to consider when managing inhibitor patients. Firstly attention is focussed on the management of acute bleeding. The options available depend on the inhibitor titre and the extent to which the patient's inhibitor cross-reacts with porcine factor VIII. The treatment choice for the patient also depends on the inhibitor titre and whether the patient is a high responder, in whom an amnestic response is likely to follow a factor VIII infusion, or a low responder, where the titre remains in the 0.5-6 BU range despite continued factor VIII exposure.

Low responder patients can be treated with factor VIII without the expectation that they will have an amnestic response that adversely affects further treatment. In some cases a temporary reduction in inhibitor titre can be achieved by plasma pheresis or immunoabsorption.

The second issue that needs to be addressed is the feasibility of reducing the inhibitor titre by inducing immune tolerance or through immune suppression. This part of the treatment may require a long period of time and can only be addressed once the acute bleeding is under control.

a) *Treatment with factor VIII concentrates*

Bleeding can be most effectively controlled when sufficient factor VIII is given to achieve a level that supports normal haemostasis .For low titre responder patients, this can be achieved by infusing increasing amounts of a factor VIII concentrate, with the dose adjusted according to the assayed response.

In general, the effects of inhibitors as 5-10 BU can be neutralised in this way. It is essential to monitor the response with factor VIII assays to be sure that an adequate level has been achieved. Once the circulating inhibitor has been neutralised the amount of factor VIII needed for subsequent infusions may be similar to that for uncomplicated haemophilia A treatment – or it may be impossibly large if a brisk amnestic response leads to enhanced antibody production.

b) *Treatment with Porcine Factor VIII*

Many factor FVIII inhibitors do not inhibit porcine factor VIII to the same extent as they do the human coagulation protein, and a high purity porcine factor VIII concentrate, Hyate: C has been used effectively in many haemophilia A inhibitor patients. It is important therefore to determine whether the patient's plasma has a low or negligible inhibitor titre when incubated with porcine factor VIII.

For haemophilia A inhibitor patients, the titre with porcine factor VIII is usually 15-30 % of that with human factor VIII, with a mean of 22%, for the 88 plasmas tested in the five largest series (Ciavarella *et al.*, 1984; Gatti & Mannucci, 1984; Kernoff, 1984; Brettler *et al.*, 1989; Morrison *et al.*, 1993). Cross reactivity is even less for most auto antibodies i.e. for 69 patients, most reported in a single large series (Ludlam *et al.*, 1994), the mean cross reactivity was only 8 %. For most patients with haemophilia A, porcine factor VIII is an important treatment option when there is

significant bleeding – or a threat of serious bleeding and the patients antibody titre has low cross reactivity i.e. the titre is less than 10-15 BU against porcine factor VIII. Ideally before porcine factor VIII is used, the cross reactivity of the patients antibody should be determined by doing an inhibitor assay utilising porcine factor VIII concentrate. If the human inhibitor titre is high, most often cross reactivity will be present and porcine concentrate will not be effective.

c) Immune Tolerance Induction

Factor VIII inhibitors may be ablated in more than 80% of selected patients with severe haemophilia A subjected to immune tolerance induction (ITI), (Mariani *et al.*, 1994, Kreuz *et al.*, 1995, Mauser-Bunschoten *et al.*, 1995). Successful ITI leads to normalisation of the factor VIII half-life, near normalisation of the patient's quality of life and a reduction in the cost of treatment. The most important predictor of successful ITI is the inhibitor titre at the start of ITI, which affects both the likelihood of success and the time taken to achieve tolerance. An inhibitor titre of less than 10BU/ml at the time of induction of ITI significantly correlated with successful outcome in both the North American (NAITR) and the International Immune Tolerance registries (p=0.004, ans 0.001))(Mariani *et al.*, 1994a; Mariani & Kroner, 1999). Tolerance may be achieved by the regular administration of factor VIII or IX over a period of time, which may vary from a few months to two or more years in resistance cases until tolerance is achieved. The consensus on which dose is best for toleration is not widely agreed. The Van Creveld regimen uses low doses of factor VIII of 25 iu/kg three times weekly (Mauser-Bunschoten *et al.*, 1995).

At the other extreme, the Bonn Regimen uses doses of factor VIII of 100iu/kg twice daily or even as high as 150iu/kg. In 1977 Brackmann and Gorsman were the first to report a curative treatment protocol for inhibitor patients using the Bonn protocol (Brackmann & Gormsen, 1977). This was based on administering high doses of factor FVIII (150iu/kg twice a day). The protocol was developed by Brackmann from 1974 to 1976 in an empirically based fashion, but the factors controlling the induction of immune tolerance toward the inhibitor antibodies and for predicting the success rate are still unknown. The high cost of the Bonn regimen has resulted in several studies using modifications of the dosage scheme.

In a study by Oldenburg et al (Oldenburg *et al.*, 1999) the Bonn protocol was successful in 60 inhibitor patients, (36 high responders and 24 low responders) a response rate of 86.7 %. In 13.3 % (8 patients) of the patients the Bonn protocol was not successful. ITI therapy was curative therapy in all successfully treated patients. No inhibitor relapse has been reported and inhibitor free intervals were documented up to 20 years. Overall the median ITI duration to achieve partial or complete success was 5.4 months and 14.1 months respectively but there was a broad range of duration of ITI up to 100 months, mainly due to patients who were treated discontinuously (one or more interruptions of ITI). These patients needed about 2-3 fold the time to achieve partial or complete success of ITI, when compared to patients continuously on ITI. Interruptions must therefore be considered as one major factor causing a prolongation of ITI and therefore should be avoided. This study also examined whether the mutation type influenced the course of ITI. In a limited number of patients the intron inversion 22 was compared to patients with other types of mutation and the ITI duration was considerably longer in those patients with intron 22 inversion.

The outcome of success and dosages is a widely disputed and contentious area. The IITR suggest that larger doses of factor VIII have a significantly better outcome in patients with inhibitor titres >10BU/ml. In contrast, neither the NAITR nor GITR were able to demonstrate that such a relationship exists. Therefore optimal dosing for immune tolerance regimens has not been agreed. Currently, there is an ongoing clinical trial to look at the outcome between the regimens (Hay *et al.*, 2001).

d) *Use of FEIBA in treatment of Inhibitors*

The management of bleeding in patients with inhibitors may use many different therapeutic modalities. Prothrombin complex concentrates and activated prothrombin complex concentrates have been successfully used in the treatment of bleeding complications in inhibitor developing patients. Despite the efficacy of these products, most physicians do not consider these activated products to be as effective as factor VIII replacement in patients without inhibitors and surgical procedures in high titre patients are often deferred. These activated or non-activated prothrombin concentrates

seem to carry some thrombogenic risk, particularly after repeated dosing such as after surgery. FEIBA (Factor Eight Bypassing Activity, Immuno, Vienna, Austria) is an activated prothrombin complex concentrate widely used in patients with congenital haemophilia who have developed inhibitors, as in acquired haemophilia. Its efficacy for the treatment of bleeding episodes varies from 64% to 88% according to differing reports (Sjamsoedin *et al.*, 1981).

e) Recombinant Factor VIIa

Recombinant FVIIA is an effective haemostatic agent that enhances the natural coagulation pathway and represents a significant advance in the management of patients with factor VIII or IX inhibitors. The unique mode of action rVIIa means it does not induce generalised coagulation. Additionally it has been shown to be non immunogenic in haemophilia A/B patients with inhibitors. Recombinant factor VIIa has been studied extensively in phase II clinical trials. The largest clinical experience included a group of 41 haemophilia A patients who have been treated with recombinant factor VIIa (Hedner *et al.*, 1993). A therapeutic effect was achieved in most patients and there were no serious side effects or evidence of systemic coagulation activation.

1.6.3 Transfusion Transmitted Infection

Although the use of plasma derived concentrates were clearly efficacious, there were hidden dangers which had not been appreciated before their widespread use and uptake. As a consequence of manufacture from large plasma pools they were contaminated with hepatitis and HIV viruses.

Hepatitis B and C

Before the development of virus inactivated factor concentrates, many patients with haemophilia became chronically infected with hepatitis B and C viruses. The high incidence of hepatitis following treatment with large pool plasma derived clotting factor concentrates was first recognised by Kasper and Kipnis in 1972 (Kasper & Kipnis, 1972). Before 1985 virtually all patients who received clotting factor

concentrate for the first time developed non A non B hepatitis (Fletcher *et al.,* 1983, Kernoff *et al.,* 1985), which has now been identified as infection with hepatitis C virus (HCV).

Since 1991, all blood donations in the UK have been tested for antibodies to hepatitis C. It has now been shown that the virus is effectively inactivated by heat and solvent detergent treatment of factor concentrates. Approximately 70% of patients infected with HCV have a chronic elevation of liver enzymes and histological evidence of hepatitis. Patients with chronic HCV infection are at an increased risk of developing hepatocellular carcinoma. A world-wide questionnaire sent to 11801 patients, with haemophilia identified a risk 30 times higher than in the general population (Colombo *et al.*, 1991). Since it is possible to cure unifocal hepatocellular carcinoma, monitoring of haemophiliac patients by ultrasound may detect this complication early on. Additionally, haemophilia patients with hepatitis C infection have a high incidence of cryoglobinaemia, which is associated with clonal B lymphocyte proliferation, and chronic hepatitis C infection may also increase the risk of B cell non Hodgkin's lymphomas.

HIV Infection

The optimistic perception of haemophilia dramatically changed in the early 1980`s when in Western Europe and North Europe 60 –70 % of patients with severe disease became infected with the human immunodeficiency virus (HIV) that had contaminated concentrates. Long-term follow up of patients with haemophilia and HIV infection has been extremely informative about the progression of the disease because, in most patients the approximate time of seroconversion is known. Large cohort studies have shown that the probablilty of remaining AIDS free using Kaplan – Meier estimates up to 25 years from seroconversion is 18% (95% confidence interval 11-25%)(Phillips *et al.*, 1994). Over the last 15 years of the last millennium, more and more efficacious virucidal methods have been developed which, have markedly reduced the risk of transmission of blood borne infections.

1.7 Gene Therapy

1.7.1 Introduction

The inherent side effects of treatment attributable to use of plasma derived concentrates, has lead to a drive to develop a better, safer, therapy for the treatment of haemophilia. Although recombinant products have reduced the risk of transfusion transmitted disease, this form of treatment is still expensive and only available in the developed world, still leaving up to 75% of the world's haemophilia population inadequately treated. A treatment, which could be administered simply and infrequently, would be the ideal in these circumstances. It is strikingly appealing that the next stage on from recombinant technology is the administration of a simple *in vivo* form of gene replacement therapy.

Concept

A conceptually new approach to the treatment of human disease has emerged during the past few decades. The concepts of gene therapy arose initially during the 1960`s and the early 1970`s. During this period cell lines were developed which could test the concept that foreign DNA could be introduced permanently, stably, functionally and heritably into mammalian cells to provide permanent new genetic functions. These studies were derived conceptually from the classical studies of Avery, McCleod and McCarty on DNA mediated genetic transformation, and since this, human geneticists have dreamed of the idea of using gene transfer to treat inherited diseases. However, it is only in the last decade that the advances made in recombinant DNA technology and cell biology have made it possible that this dream may become a reality. There are two main approaches, somatic and germline modification.

Somatic gene therapy: Any cells other than germ cells may have their genetic constitution altered. Therefore this only affects the individual's genetic constitution during their lifetime and would not affect their children.

Germline gene therapy: Alternatively, foreign genes can be injected into fertilised eggs. The inserted genes would be distributed amongst somatic and germ cells and will be permissive to transmission to future generations.

For a variety of technical and ethical considerations, current research is therefore restricted to somatic cell gene therapy. All the work described in this thesis is directed towards a somatic approach.

Definition of Gene Therapy

Gene therapy may be defined as the introduction of nucleic acids into cells for the purpose of altering the course of a medical condition or disease. In general, nucleic acids are DNA molecules, encoding gene products, or proteins. Although the simplest concept of gene therapy is for the treatment of monogenic gene disorders, it is possible that gene therapy can be considered as a new pharmaceutical tool for the treatment of many types of disease which have, as part of their aetiology, a genetic basis. There are many candidate diseases for a gene therapy approach, both genetic and acquired. In order to be considered for this approach, knowledge of the basic biology and science of the disease in question is required.

In particular, it needs to be understood that the phenotype of a particular disease can be readily explained by genotypic defect. In the case of haemophilia it is known that certain disease mutations result in a qualitative or quantitative defect of the respective clotting factor protein, resulting in an absence of circulating protein and haemophilia (*see earlier*). Candidate disorders where disease phenotypes manifest from the underlying genetic defects are shown in the table below. Theoretically, all these disorders may one day be amenable to a gene-based approach once the technology is optimised.

Table 2 *Candidate genetic diseases amenable to a Gene Based approach*

Disease	Defect	Incidence	Target Cells
Severe combined Immunodeficiency (SCID/ADA)	Adenosine deaminase	Rare	Bone marrow cells T lymphocytes
Haemophilia A,B	Clotting factor VIII or IX deficiency	A: 1: 10 000 B: 1: 30 000	Liver ,Muscle, Fibroblasts, Bone Marrow Cells
Familial Hypercholesterola-emia	Deficiency of low density lipoprotein receptor	1:1000000	Liver
Cystic Fibrosis	Loss of CFTR gene	1:3000	Airway Epithelial Cells
Haemoglobinopathies Thalassaemias/ Sickle cell disease	Structural defects in α/β genes	1:600	Bone marrow cells
Gaucher`s disease	Enzyme Glucocerbrosidase	1:450	Bone marrow cells
α_1 anti trypsin deficiency	Lack of anti trypsin	1:3500	Lung or Liver cells

1.7.2 Experimental considerations to address before development of gene therapy protocols

In the consideration of a gene therapy treatment for hereditary disease, several processes have to be gone through and evaluated before it is possible to move into a clinical setting. First, the nature of the genetic defect leading to the disease in question should be completely understood. This will allow the appropriate cloning of the normal genes in question so they can be incorporated into prospective gene delivery vehicles. Equally important, is a detailed knowledge of the biology of the disease in question i.e. is the absence of a particular protein directly correlated with the manifestations of the disease in question? Will replacement of the missing protein ameliorate the disease manifestations? This knowledge is required so that appropriate target cells are selected for a gene-based approach.

This knowledge forms the basis of testing out the proof of principle for a gene-based approach i.e. *in vitro* transfection of appropriate target cells with expression of a

biologically active protein product. Once *in vitro* efficacy has been established, the next phase of experimentation is the extrapolation of *in vitro* findings to an *in vivo* model. This will require the use of animal models of the disease in question to test the feasibility of the approach. Finally, once safe *in vivo* efficacy has been established, the stage is then set for testing out in human subjects. The scheme of testing is therefore shown as follows:

	***Invitro* testing**	***Invivo* testing**
Considerations	Target cell choice	Genes cloned (homologous)
	Vectors	Animal model available?
	Biologically active protein?	i.e. large and small model
		Safety?
		Toxicity?

Once these criteria have been met, and proof of principle has demonstrated in a safe and non-equivocal fashion, it is then safe to proceed to further testing in a clinical setting involving human subjects.

1.8 Haemophilia and Gene Therapy

1.8.1 Introduction

The concept of gene therapy treatment for haemophilia first came about in the late 1980`s. The possible use of a gene-based approach to treat haemophilia has been facilitated by the advances made in molecular biology over the last 20 years, which began with cloning of the clotting factor genes and which resulted in the capacity for advances in technology to develop recombinant clotting factor concentrates.

The relative disadvantages of protein-based therapy have fuelled interest in gene therapy for haemophilia. Gene therapy would potentially allow patients to realise the benefits of prophylaxis, including a reduced incidence of intracereberal haemorrhage and of haemophilic arthropathy, without the need for indwelling intravenous catheters

or frequent factor infusion. The major advantage of such an approach would be that a constant level of clotting factor is maintained so that bleeds are prevented, and thereby form a safeguard not only against chronic joint injury but also against potentially fatal bleeding episodes such as intracranial haemorrhage. Thus it is possible that the relative 'cure' of haemophilia may be one of the first genetic therapies to evolve from molecular scientific advances made in the 20th century.

1.8.2 Advantages of a gene based approach for haemophilia

Haemophilia is potentially a very attractive model for a gene-based approach for a number of reasons :

1) Wide range of suitable target tissues with access to the circulation.

Clotting factors are normally synthesised in the liver, but several investigators have shown that biologically active clotting factors may be produced in several cell types, including fibroblasts (Palmer *et al.*, 1989), myoblasts, (Yao *et al.*, 1991), endothelial cells (Yao *et al.*, 1991) and keratinocytes (Gerrard *et al.*, 1993). These initial studies were important, since they provided the initial platform for proceeding from *in vitro* studies to proof of efficacy in animal models of disease. Therefore, the choice of target cell is not limited and the transgene can be inserted into any one of a number of tissues so long as the transgene product can gain access to the circulation.

2) *Precise regulation of the transgene expression is not required.*

A second advantage of haemophilia as a model for gene therapy is that precise regulation of expression of the transgene is probably not required. Most patients with haemophilia have severe disease with less than 1u/dl. Patients with levels greater than 5u/dl rarely experience spontaneous bleeding episodes (moderately affected).

Therefore, raising a patients level from >1u/dl to <5u/dl will result in amelioration of a severe disease phenotype. Similarly, it is clear from data based on infusion of clotting factor concentrates into patients with haemophilia that levels as high 150u/dl are not associated with ill effects since the proteins circulate as zymogens. Therefore,

a remarkably wide range of levels of expression (3-150u/dl) falls within the therapeutic range. The situation in haemophilia is thus different from other medical disorders characterised by enzyme deficiency such as diabetes mellitus where loss of tight regulation of the process may lead to deleterious effects i.e. hypoglycaemic coma.

3) Clear endpoints for efficacy available

For haemophilia this is straightforward and the following may assess the efficacy of the procedure

a) *In vitro* assays: Clotting based assays to determine correction of clotting, measurement of circulating clotting factor levels by clotting assays and Elisa assays.
b) Observation of clinical phenotype and bleeding frequency
c) Coagulation factor usage

4) Availability of animal models

A fourth advantage compared with other diseases, is the availability of small and large animal models that faithfully mirror the human disease. This has been facilitated by the cloning of the relevant species cDNA and the subsequent use in gene knockouts to produce clinically affected murine models of both Haemophilia A (Bi *et al.*, 1995) and Haemophilia B (Lin *et al.*, 1997, Wang *et al.*, 1997). Additionally there are well-characterised naturally occurring canine models of haemophilia (Evans *et al.*, 1989, Brooks *et al.*, 1997).

The development of animal models of haemophilia is essential to the development of a gene therapy protocol for two main reasons: to test the feasibility of a gene based approach in an animal model, and to demonstrate that scale up is achievable from a small animal model such as a mouse to a large animal model such as a canine one.

1.8.3 Approaches to Gene therapy for haemophilia

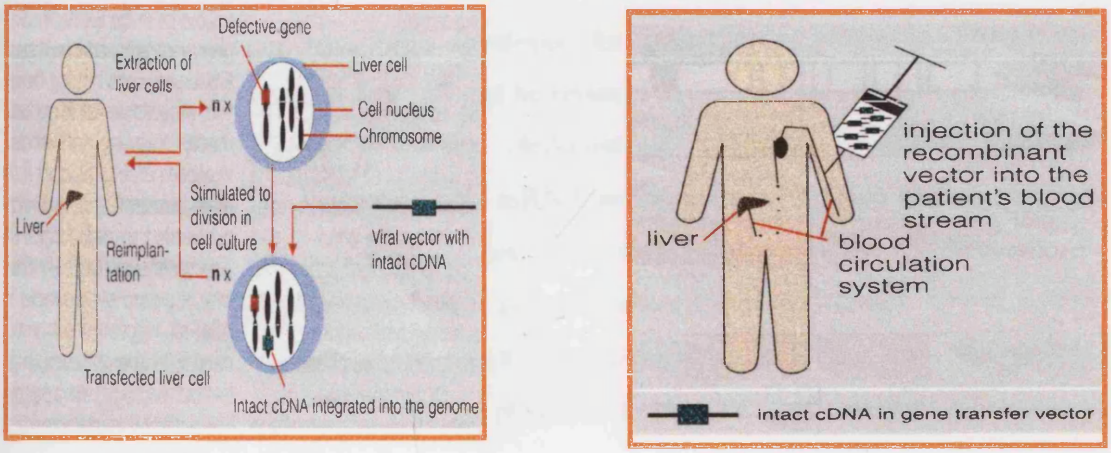
The introduction of the genetic material for factor VIII or factor IX into the appropriate targets is the only definitive approach to attempt to cure haemophilia. The genes may be delivered into target cells by two groups of vehicles: a) using recombinant viral vectors or b) using physical gene delivery methods. The requirements for a gene-based approach are easy to define, but are technically very demanding to achieve. The gene (or cDNA) is placed under the control of a strong (usually) viral promoter/enhancer, which may be modified to be specific for the tissue in which it is desired to express the gene.

Gene Delivery

Two approaches may be taken for the delivery of genes. In the *ex vivo* approach the gene of interested is introduced into cells in tissue culture and then transplanted back into the host animal. Alternatively the vector may be introduced into the recipient directly (*in vivo* approach). This approach does not generally attempt to target specific tissues.

Figure 7 *Ex vivo and In vivo approaches*

Ex Vivo ***In Vivo***



1.8.4 Ex vivo approaches

The genetic modification in this approach is by transfecting cells to produce the desired protein in tissue culture, and then returning the functional cells to the patient. *Ex vivo* modifications allow for the optimisation and control of gene transfection. Additionally, unmodified cells can be eliminated prior to transplantation. The return of genetically modified cells to the body may be difficult. For the return of transduced haemopoietic cells, the process is straightforward with return to the blood stream. However, for other cells it may be necessary to establish a supporting matrix and blood supply to sustain the implanted cells and deliver the desired protein to the bloodstream. Facilitation of efficient transplantation of *ex vivo* cells may be achieved by the use of capsules with semipermeable membranes (Kingdon *et al.,* 1993).

1.8.5 In vivo approaches

The alternative to *ex vivo* transfection is the direct modification of cells in the body (*in vivo* modification). The advantages of this approach are the simplicity, the ease with which it might be applied to patients and the fact that it eliminates the problem associated with transplantation of cells. This form of therapy would be the ideal, but it would need to be highly efficient, targeted, and not inactivated *in vivo* by inducing an immune response.

1.9 Choice of Gene Delivery Vehicle

Several DNA vectors have been developed for gene therapy protocols. The vector must have the ability to enter a cell and be retained in the nucleus either as an episome (a separate DNA entity from the chromosomal DNA) or integrated into the chromosome, and be transcribed into mRNA in the nucleus. Vectors may be divided into viral vectors and non-viral vectors. Viral vectors have evolved efficient natural mechanisms of infecting cells such as retroviruses (Vile & Russell, 1994), adeno viruses and adeno-associated viruses (Kremer & Perricaudet, 1995). Non viral vectors (Shcofield & Caskey, 1995) such as plasmids or plasmid lipid mixtures (liposomes)

which are artificially introduced into cells, or plasmid protein complex can bind to receptors on the surface of cells and enter by endocytosis.

Non Viral Vectors

Non-viral vector systems vary from direct injection of DNA to the mixing the DNA with polysine or cationic lipids that allow the gene to cross the cell membrane. Most of these approaches suffer from low efficiency of transduction and transient expression of the transgene due to episomal location within the nucleus. In relation to the haemophilia B gene therapy studies, factor IX containing plasmids have been electroporated into liposomes (Baru *et al.*, 1995) and injected intravenously into mice. They have also used been to *ex vivo* modify myoblast cell lines (Hortelano *et al.*, 1996) and bone marrow stromal cells (Hurwitz *et al.*, 1997) before reintroducing the transduced cells into mice or dogs. Both of these approaches have yielded low and transient levels of factor IX.

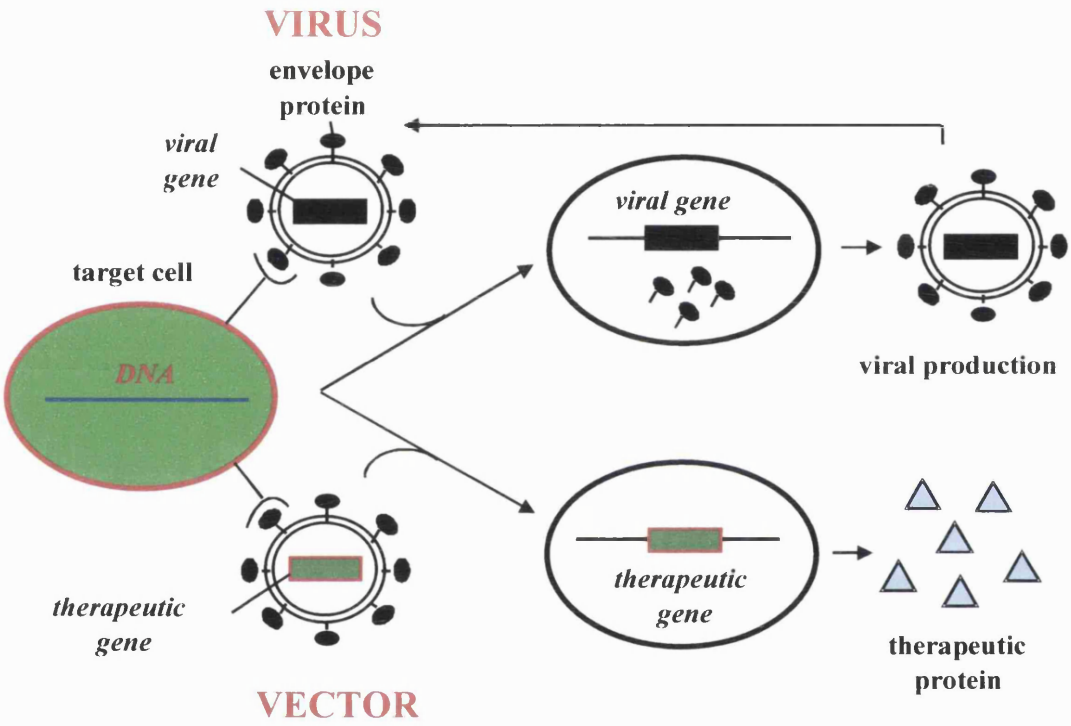
More recently *in vivo* site directed mutagenesis using chimaeric RNA-DNA oligonucleotides has been reported to efficiently and specifically introduce single nucleotide changes in codons to the factor IX gene in rat hepatocytes, thereby changing the coagulation activity in plasma samples (Kren *et al.*, 1998). For correction of a genetic defect, this strategy requires that the mutation be characterised and that it be a point mutation. For all the non-viral strategies, further studies in large animals will be required to assess how competitive they are when compared with viral approaches.

Viral

Viral vectors include retroviral, lentiviral, adenoviral and adeno-associated vectors. Each vector system has its own advantages and limitations. Common to all the virus systems is the ability of each vector to transfer genetic material as part of the infectious process. In general, viral vector mediated gene transfer is more efficient than non-viral gene transfer, and at present is the method of choice. Viruses can bind to cellular receptors and introduce their genetic material into cells. When the viral genes are expressed in the target cell, new viral particles can be generated triggering a productive infection. Vectors may therefore exploit the same entry mechanisms as

viruses from which they were derived. However, in vectors, some if not all of the viral genes required for replication have been replaced with potential therapeutic transgenes such as factor VIII or IX. Consequently, vectors cannot replicate within their target cell, and cannot initiate a productive infection but express the therapeutic protein instead (*see figure 8*).

Figure 8 *Viral Infection of target cells*



Retroviruses

Structure

Moloney murine leukaemia virus based retroviral vectors (MoMLV) have been the most commonly used vectors for gene therapy approaches. The RNA in the viral genome is converted to DNA in the infected cell. The genome comprises three genes termed *gag, pol,* and *env*, which are flanked by elements called long terminal repeats (LTRs). These are required for integration into the host genome and they also define the beginning and the end of the viral genome.

The LTRs also serve as enhancer-promoter sequences and control the expression of viral genes. The final element of the genome, the packaging sequence (ψ), allows viral RNA to be distinguished from other RNAs in the cell. By manipulation of the viral genome, viral genes can be replaced with transgenes such as factor VIII and IX. Transcription of the transgenes may be under the control of the viral LTRs or alternatively, enhancer promoter elements can be engineered in with the transgene. The chimaeric genome can then be introduced into a packaging cell, which produces all of the viral proteins (such as the products of the *gag, pol, env* genes). However, as these have been separated from the LTRs and the packaging sequence, only the chimaeric viral genomes are assembled to generate a replication-incompetent retroviral vector.

Advantages

These vectors can be produced at moderate titres in vector producer cell lines and have the capacity to transduce a wide variety of cell lines. Therefore *in vivo,* if the given target cells transduced can produce both active clotting factor and secrete it into the circulation, delivery of the vector during the appropriate part of the cell cycle should provide physiologically active clotting factor into the circulation. As the vectors integrate into the host cell genome, progeny cells will also express the vector sequence. Therefore, this allows long-term maintenance of the vector in the treated individual, and ensures that only a few transduced cells provide satisfactory levels of clotting factor.

Compared to adenoviral vectors (*see below*), retroviral vectors are relatively non-immunogenic, and do not directly induce immune responses to viral components or induce any adjuvant effects (McCormack *et al.*, 1997). Significant advances have been made in the production of retroviral vectors with the development of high titre retroviral producer cell lines, leading to an increase of about 100 fold over those titres previously available and making production for human subjects attainable. To date there has been considerable experience with these vectors, and now well over 1500 patients have been treated with these vectors giving increasing confidence in overall safety and clinical acceptability of these agents (Anderson, 1998).

Disadvantages

Since these vectors integrate, there is potential for insertional mutagenesis resulting in possible oncogenic activation. Although this possibility cannot be excluded, so far in more than 1500 patients treated in clinical trials, no integration has been seen (Anderson, 1998). Special care will need to taken in the production of these vectors to ensure that replication competent virus is eliminated from the final product. Clinical batches of retroviral vector are stringently tested for replication competent retroviruses (RCR). New packaging cell lines are engineered to reduce homologous regions that might lead to homologous recombination. Another potential concern for retroviral vectors is their susceptibility to inactivation by primate complement (Takeuchi *et al.*, 1996). To this end, newer packaging cell lines are being developed to produce vector that is resistant to primate complement inactivation (Greengard & Jolly, 1999).

Uses of Retroviruses in Haemophilia

Haemophilia B

Because of their potential to transduce several different target cells and produce biologically active factor IX, a number of *ex vivo* approaches using these cells have been tried. These included primary fibroblasts (St Louis & Verma 1988, Palmer *et al.*, 1989, Axelrod *et al.*, 1990), hepatocytes (Armentano *et al.*, 1990), endothelial cells (Yao *et al.*, 1991), and myoblasts (Dai *et al.*, 1992a, Yao *et al.*, 1994). Some early success was achieved transfecting murine myoblasts, with factor IX being expressed for up to six months, albeit with low levels. The reason for this was due to the host immune response (Yang *et al.*, 1994b) and inactivation of the viral promoters (Yang *et al.*, 1994b).

Studies were then extended into larger animals, but the results were even less successful, with levels being achieved which were clinically insignificant (unpublished observations). However, due to the initial promise of these studies, a human trial was undertaken in China using fibroblasts as the target cell which were subsequently transplanted subcutaneuosly back into two boys. One boy was reported

to have an increase in factor IX activity with a decrease in requirement for replacement therapy whilst the other boy did not achieve any benefit (Lu *et al.*, 1993).

Subsequent *in vivo* approaches were tried but with little success. In this case using portal vein infusion, a retroviral vector expressing canine factor IX was infused via a splenic catheter. To induce cell division this animal underwent a two-thirds hepatectomy to enable the retrovirus to infect the target hepatocytes. This procedure produced only low levels of factor IX (0.1u/dl) for 9 months (Kay *et al.*, 1994). Clearly, this approach would be impratical and unacceptable in humans.

Haemophilia A

Ex vivo Approaches

Early progress with factor FVIII was problematic because of the presence of a transcriptional silencer (Hoeben *et al.*, 1995) or an inhibitory sequence (Koeberl *et al.*, 1995). This has been mapped to two independent regions of the A2 region of the factor VIII coding sequence (Lynch *et al.*, 1993). Despite mutation of these sequences, factor VIII expression failed to increase (Hoeben *et al.*, 1995). However, subsequent reports (Chuah *et al.*, 1998) showed that incorporation of an intron upstream of the factor VIII coding sequence lead to considerable improvement in vector titre and factor VIII expression.

Initially, several *ex vivo* approaches were tried using fibroblasts (Hoeben *et al.*, 1992a; Hoeben *et al.*, 1992b) and bone marrow cells (Hoeben *et al.*, 1993). However, this approach did not yield significant circulating levels of factor VIII. An improvement was made by Dwarki et al (Dwarki *et al.*, 1995) using an MFG viral backbone to transduce fibroblasts, which were then implanted as neo-organs into SCID mice resulting in transient (one-week) expression of factor VIII. The potential for using skin as a target cell has been clealy shown, and further studies have shown skin transplants from doubly transgenic mice that expressed factor VIII only under a dermis specific involucrin promoter were able to reconstitute normal clotting in Rag-1/ FVIII deficient double knockout mice (Fakharzadeh *et al.*, 2000).

Other *ex vivo* cell targets were also tried, such bone marrow stromal cells (Chuah *et al.*, 1998) which, following transplantation into immunodeficient mice produced factor VIII activity of 20ng/ml.

In vivo Approaches

For this approach, hepatocytes are the favoured target cell but high titres of vector are required. A major technological barrier to the initial use of gene therapy vectors was the lack of technology, which could produce recombinant vectors in sufficient titre to enable high levels of gene expression to be attained. Considerable technological advances have been made in vector production and purity, enabling titres of virus to be produced several fold higher than first generation vectors, enhancing the desirabilty of the liver directed route.

Other advances in the liver route have been the use of antecedent infusion of hepatocyte growth factors that increase the number of cycling hepatocyte cells to 5-13% and increase the number of transduced cells to 1-2 % (from untreated baseline 0.1%) (Bosch *et al.*, 1996; Patijn *et al.*, 1998a; Patijn *et al.*, 1998b). The tropism of these vectors has been improved by the use of pseudotyping of the vector glycoprotein coat with a vesicular stomatitis envelope (VSV). Vanden-Driessche (Vanden-Driessche *et al.*, 1999) reported a significant advance in the use of these vectors, demonstrating that therapeutic levels of factor VIII in newborn mice could be reached using a pseudotyped retroviral vector (VSV). Six of 13 mice expressed factor VIII, with four mice expressing at levels higher >50% normal human plasma levels. The remaining mice developed antibodies to the non-species specific transgene. Importantly in these mice, expression was maintained for greater than 12 months. The high levels of expression observed in these studies may have been influenced by the age of the mice (newborn animals) in which the hepatocytes are in high rapid proliferation. These results bear out those of Greengard et al (Greengard & Jolly, 1999) in larger animals (rabbits and dogs), where long term expression of factor VIII was obtained for six months.

Adenovirususes

Human adenoviruses are responsible for mild illnesses such as respiratory infections. The more common serotypes 2 and 5 have been exploited for use in clinical gene therapy trials for cystic fibrosis and cancer (Wilson, 1993). The 36-Kb double stranded DNA virus contains genes that express more than one gene product during the life cycle. Like other DNA viruses, two main phases are distinguished during the course of an infection: an early phase with the expression of the early viral genes E1 to E4, and a late phase, after the onset of viral replication, characterised mainly by the production of structural proteins. A lytic infectious cycle requires two to three days, during which 1000 to 10000 infectious particles are produced per cell (Kochanek, 1999).

By eliminating the E1 region of the vector, two goals are accomplished: space is made for the placing of gene sequences and in the absence of the E1a protein the virus cannot replicate. The first generation adenoviral vectors have been shown to be very efficient at transferring genes into most tissues after *in vivo* administration. These vectors can transduce non-dividing cells and do not integrate into host DNA. These vectors have been shown to effect a temporary cure in a number of animal models with diseases like haemophilia and hypercholesterolaemia (Jaffe *et al.*, 1992), (Kozarsky *et al.*, 1993; Connelly & Kaleko, 1998). However, it was subsequently shown that low-level production of viral antigens from the vector elicited a strong immune response that eliminated the transduced cells and the transgene product (Yang *et al.*, 1994b; Yang *et al.*, 1994c). Also, re-administration was not possible due to the immune response (Kay *et al.*, 1997).

The immune response was potent at eliciting both a cytotoxic cellular response and an antibody producing humoral response. In the cellular response the virally infected cells were killed by cytotoxic T lymphocytes. The humoral response resulted in the generation of antibodies to adenoviral proteins and it prevented any subsequent infection if the animal was given a second injection of the recombinant adenovirus. In order to overcome the problem of the immune response, second generation adenoviruses were developed in which additional genes implicated in viral replication were deleted.

The idea has now been taken further with gutting the vectors (Fisher *et al.*, 1996a), deleting all the viral genes, leaving only the beginning and the end of the genome and the viral packaging sequence. The transgenes encoded by these viruses persisted up to 84 days (Fisher *et al.*, 1996a). However even if the genes are removed there is still some level of immunity to the transgenes. Immunomodulatory agents that are selective may be useful in blocking these immune responses as demonstrated in several animal models (Wilson & Kay, 1995) (*vide infra Chapter 7*).

Adenoviruses in Haemophilia A

Adenoviral vectors have a lot of features, which make them attractive as gene delivery vehicles especially for hepatocytes. They are able to be produced in high titre, have broad infectivity, potential for a large payload, and have *in vivo* gene delivery capacity (Zhang *et al.*, 1998). Intravenous administration of B domain deleted constructs to mice dogs and primates have resulted in efficient transduction of liver tissue (Connelly & Kaleko, 1998), (Connelly *et al.*, 1996a; Connelly *et al.*, 1998; Connelly & Kaleko, 1998; Gallo-Penn *et al.*, 1999). High-level factor VIII expression has been noted in mice with sustained phenotypic correction (Connelly *et al.*, 1998). In some mice, correction lasted longer than 12 months with levels of greater than 2000mu/ml. When these studies were extended to haemophilic dogs, expression was short lived in the main due to a humoral response to human factor the VIII (Connelly *et al.*, 1996b). In studies with human primates when a tagged FVIII construct was used, therapeutic levels of factor VIII expression were obtained but these were short lived and only recorded at the high vector doses (Brann *et al.*, 1999). Transient elevation of liver enzymes was noted, with evidence of liver inflammation by histology.

The induction of a host immune response to these vectors has fuelled an interest in the development of vectors, which are devoid of all the viral coding sequences responsible for the immune response. These so called "gutless" vectors have resulted in the *MiniAd* vector constructs, which contain the minimal *cis* elements that are required, and contain a full length FVIII cDNA under the control of an albumin promoter (Zhang *et al.*, 1999). When injected into mice via the tail vein, these vectors

encoding the full-length factor VIII cDNA, resulted in physiological levels of circulating factor VIII (100-800 ng/ml in 3/16 mice) sufficient to correct the haemophilic phenotype. Expression gradually declined, due to the presence of anti human factor VIII antibodies (Balague *et al.*, 2000).

Adeno–associated virus (AAV)

AAV vectors are engineered from human AAV, a non-pathogenic, replication defective parvovirus. Wild type AAV is composed of a single stranded DNA genome of 4800 nucleotides. The virus has both lytic and latent phases in its life cycle. The lytic phase requires a helper virus e.g. adenovirus or herpes virus. In the absence of a helper virus, wild type AAV integrates into the host cell genome. AAV integration shows site specificity with high proportion of events occurring at a specific site on chromosome 19.

To generate recombinant vector from the virus, the viral coding sequences are replaced by the gene of interest in a plasmid containing only the viral inverted terminal repeats (ITRs), and the necessary viral genes supplied in *trans* on a second plasmid. Following introduction of these plasmids into 293 cells, the cells are infected with helper adenovirus and lyse 48 hours later, and recombinant AAV is purified from the cell lysate (Samulski *et al.*, 1989, Snyder *et al.*, 1993)

Most of the AAV vectors studied to date are based on the AAV-2 serotype. Since most humans are seropositive for AAV-2, it is possible that the presence of these AAV specific antibodies would interfere with AAV transduction *in vivo*. To circumvent this problem, other AAV serotypes could be used. The large scale production of high titre preparations of AAV is laborious and difficult, and efforts to enhance this are being developed to generate stable packaging cell lines by further improving vector purification strategies using high affinity chromatography (Clark *et al.*, 1999; Anderson *et al.*, 2000; Auricchio *et al.*, 2001; Rabinowitz *et al.*, 2002).

Replacing the structural and regulatory genes, *cap* and *rep*, with factor VIII or IX, produces the recombinant vector, rAAV. These inserts are flanked by two inverted terminal repeats (ITRs) which are the minimal *cis* elements necessary for integration,

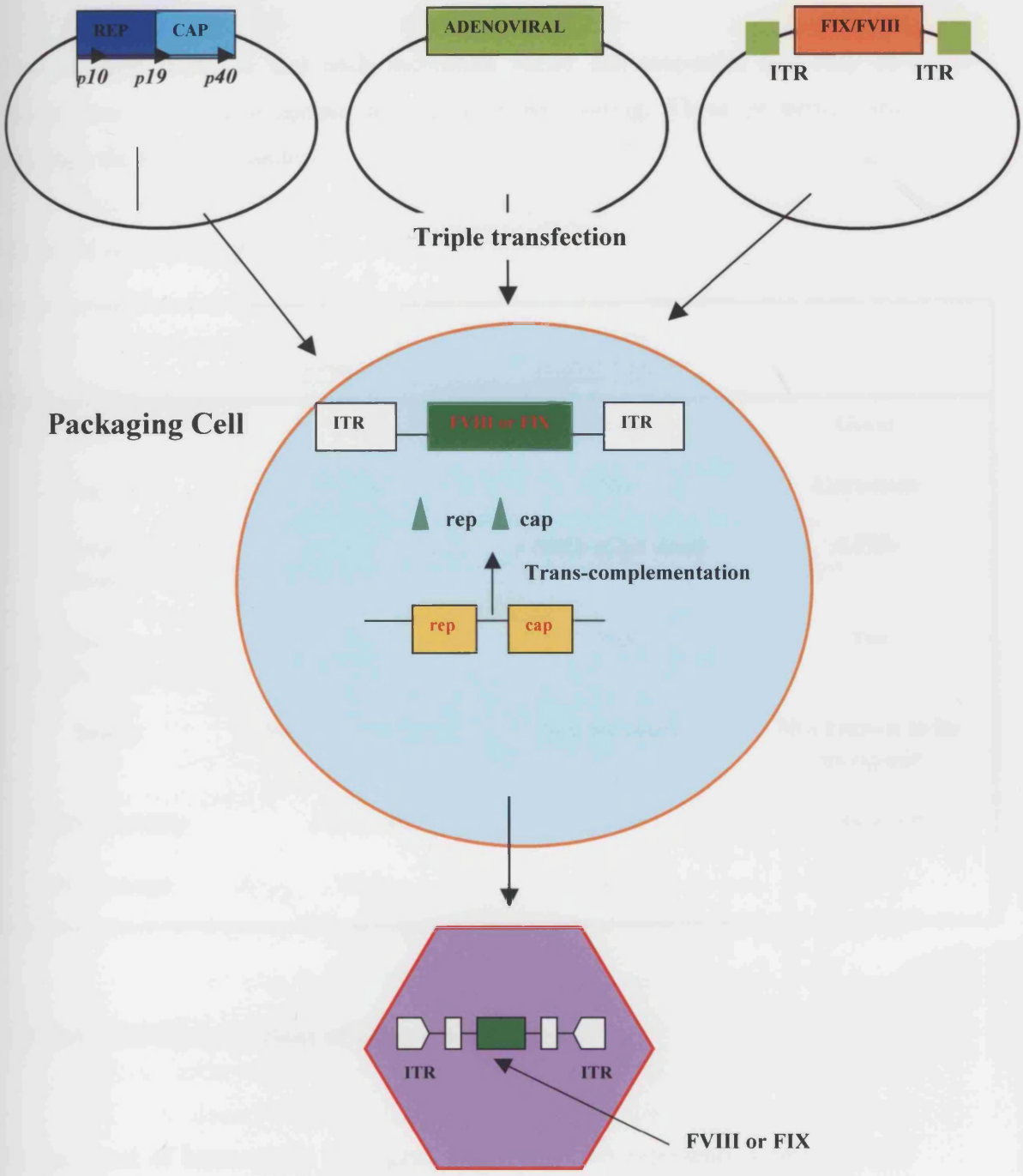
replication, encapsulation and rescue from the host cell genome. The wild type virus is not pathogenic in humans and is replication defective requiring a helper virus such as adenovirus to generate infectious particles. The outline of the process is shown in *Figure 9.*

The vector itself seems to have a distinct number of advantages, making it useful for gene therapy applications. Firstly, it has the ability to transduce non-dividing cells, such as brain, muscle and liver (Blau & Khavari, 1997, Fisher *et al.*, 1997, Kaplitt *et al.*, 1994, Kessler *et al.*, 1996, Snyder *et al.*, 1997, Xiao *et al.*, 1997).

Secondly, removal of all the viral coding sequences prevents the generation of wild type helper virus and eliminates the possibility of immune responses to residual viral genome expression. In place of the 4.4 KB coding region, the virus can carry and express non-viral genes up to 5Kb. Similar to Adenovirus, rAAV can infect both dividing and non-dividing cells.

Thirdly, and crucially for haemophilia, it appears that when the vector is used as a gene transfer vehicle, the length of expression achieved is good. An initial report using rAAV lac Z containing the bacterial β galactosidase gene demonstrated stable muscle based expression in Balb C mice for at least 32 weeks after IM administration of the vector. (Kessler *et al.*, 1996). Long-term persistence of β galactosidase protein expression was demonstrated for greater than 1.5 years in healthy mice (Kessler *et al.*, 1996). A highly significant finding in this early work was the fact that rAAV did not induce substantial cell mediated immune responses. It was found that the rAAV vector did not elicit a CTL response to the vector transduced cells and in particular to the β galactosidase. These results are in stark contrast to Adenoviral vectors using the same transgene (Yang *et al.*, 1996a). This result would be very desirable for using a secretable transgene such as factor IX.

Figure 9 *Generation of Recombinant AAV vectors*



AAV –FactorVIII or AAV-Factor IX vector

To generate recombinant AAV vector particles, human or primate cells (HeLa, 293 or COS) are first transfected with the recombinant AAV vector DNA and a complementing plasmid, which supplies the Cap and Rep proteins, but that cannot be packaged into AAV virions because the ITRs are missing. The transfected cells are subsequently infected with helper adenovirus, which initiates a productive lytic infection, or transfected with a plasmid encoding the essential adenoviral genes required for AAV replication

Summary

It can be seen therefore that each individual vector has properties that may be advantageous or disadvantageous in a gene based setting. These properties are outlined in the following table:

Table 3 *Comparison of vectors*

	Retroviral	Adenoviruses	Adeno-associated
Titre	Low	High	Good
Integration	Yes	No	Uncertain
Maximum insert size	~ 6Kb	> 30Kb (Gut -less)	4.7Kb
Ability to enter non dividing cells	No	Yes	Yes
Safety	Possibly oncogenic	Not oncogenic	Not known to be oncogenic
Vector stability	Moderate	Good	Very good
Host range	Wide	Wide	Wide

1.9.1 Potential Complications of a Gene Based Approach

The treatment of haemophilia by a gene based approach represents a new form of treatment for the disease and brings with it new concerns about the possible complications of treatment. The complications of a gene transfer therapy centre around three main issues:

1) *Inadvertent germ line transmission*
2) *Insertional mutagenesis*
3) *Inhibitor formation*

1) ***Inadvertent Germ line Transmission***

Here, there exists the possibility that the injected vectors instead of just inserting into their target tissues could theoretically disseminate inadvertently to the germline tissues of recipients. This could result in the transmission of the donated gene sequences to subsequent generations. If this were to result in permanent correction of the genetic defect this would be useful, but because most vectors integrate randomly, concern exists that the donated gene sequences may result in harm to the offspring i.e. if the site of integration disrupts a critical gene sequence for the developing embryo or if expression of the donated gene somehow disrupts the normal program of development.

Therefore, part of the work up for any gene-based strategy is an assessment of the likelihood that the donated gene sequences will be transmitted to future generations. Generally, the risks are lower for *ex vivo* strategies than *in vivo* strategies but this possible complication needs to be explored in pre-clinical studies prior to undertaking clinical studies.

2) Insertional mutagenesis

The risks of random insertion of integrating vectors into the host genome may lead to severe deleterious consequences such as the activation of proto-oncogenes and oncogenes which may alter the neoplastic propensity of any given cell. This could result in devastating long-term neoplastic complications of a gene-based approach. Data specifically addressing this point continue to accrue as the number of long term survivors of gene therapy approaches with integrating vectors increases (Ye *et al.*, 1998).

Ideally, integration into the host cell genome should be ordered and predictable (to avoid oncogenicity) to allow for long-term production of the gene product when the target cell undergoes division. A useful approach to adopt for the time being until more data becomes available would be to limit initial trials with integrating vectors to older subjects who have less life expectancy. Such an approach would allow accrual

of more data on the effects of integrating vectors while protecting the population that would be most affected by late appearing complications.

3) ***Formation of Inhibitory antibodies***

An important issue facing all gene therapy trials for haemophilia is the risk of forming antibodies to the transgene product. The formation of neutralising antibodies is the most common complication of protein-based replacement therapy, occurring in approximately 20% of patients with haemophilia A and 3% of those with haemophilia B (Brettler, 1996; Kay & High, 1999). Despite several years of study it is still not possible to predict which patients will develop inhibitory antibodies, but certain risk factors have been identified, such as the nature of the underlying mutation, inherited characteristics of the individuals immune response, and the circumstances surrounding exposure to the clotting factor protein i.e. the presence of tissue injury or inflammation.

A major concern for a gene-based approach is, what will the incidence of inhibitor formation be, using such a novel therapy? At the initiation of gene therapy protocols in the mid 90`s no study had addressed these issues. In particular, the mechanism of inhibitor formation in this approach is important since knowledge of this will facilitate intervention strategies to deal with this complication. Factors that are likely to influence inhibitor formation in a gene based approach include the vector itself, target-tissue selected, and inclusion of tissue specific promoter elements.

Another important consideration for a gene based approach, and the development of inhibitor formation is the mode of antigen delivery to effect satisfactory therapeutic clotting factor levels. In the conventional protein based approach, delivery of antigen is by intermittent intravenous infusion. This in turn will lead to peak and trough levels of infused protein and the relationship of this situation to inhibitor formation is not well understood. However, for a gene-based approach the situation is different, since gene expression from the target tissue is constant, protein expression will be continuous. The effect of this on inhibitor formation will be different, from the protein-based situation, and some investigators have argued that continuous expression of antigen may be more tolerogenic, leading to less inhibitor formation,

and that a gene based approach may even be used as a treatment for inhibitor formation (Evans & Morgan, 1998).

The mechanism of inhibitor formation and immune responses in a gene-based approach requires characterisation. The emphasis and objectives of this thesis is the characterisation of the immune responses and the possible development of inhibitory antibodies and strategies to modify such responses. These issues are expanded upon in *chapter 2*, which outlines the problem and strategies to tackle it.

CHAPTER 2

INITIAL AIMS AND OBJECTIVES

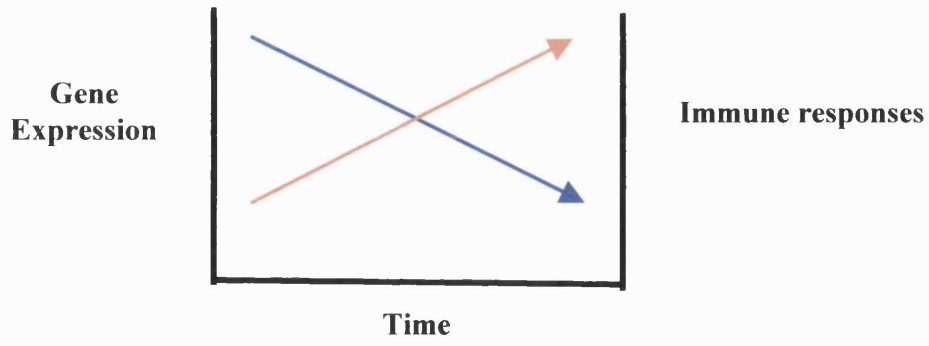
2.1 The Immune Response to a Gene Transfer Approach

The problem

One of the biggest concerns for a gene-based approach for the treatment of a human disease is the development of an immune response to the process. This happens in the conventional approach for haemophilia, where an immune response results in a antibody mediated humoral response to the infused clotting factor protein. Clinically, such inhibitory antibodies (inhibitors) complicate therapy, and can make affected patients very difficult to treat.

The occurrence of an immune response may be anticipated, since the introduction of any new protein (or transgene product) will be seen as foreign by a naive host and therefore trigger an immune response. For a gene-based approach to be successful, gene expression must continue and not be down regulated by an immune response. If this were to occur, gene expression may be lost and the treatment aims fail. *Figure 10* stylises the appearance of an immune response and consequent loss of gene expression.

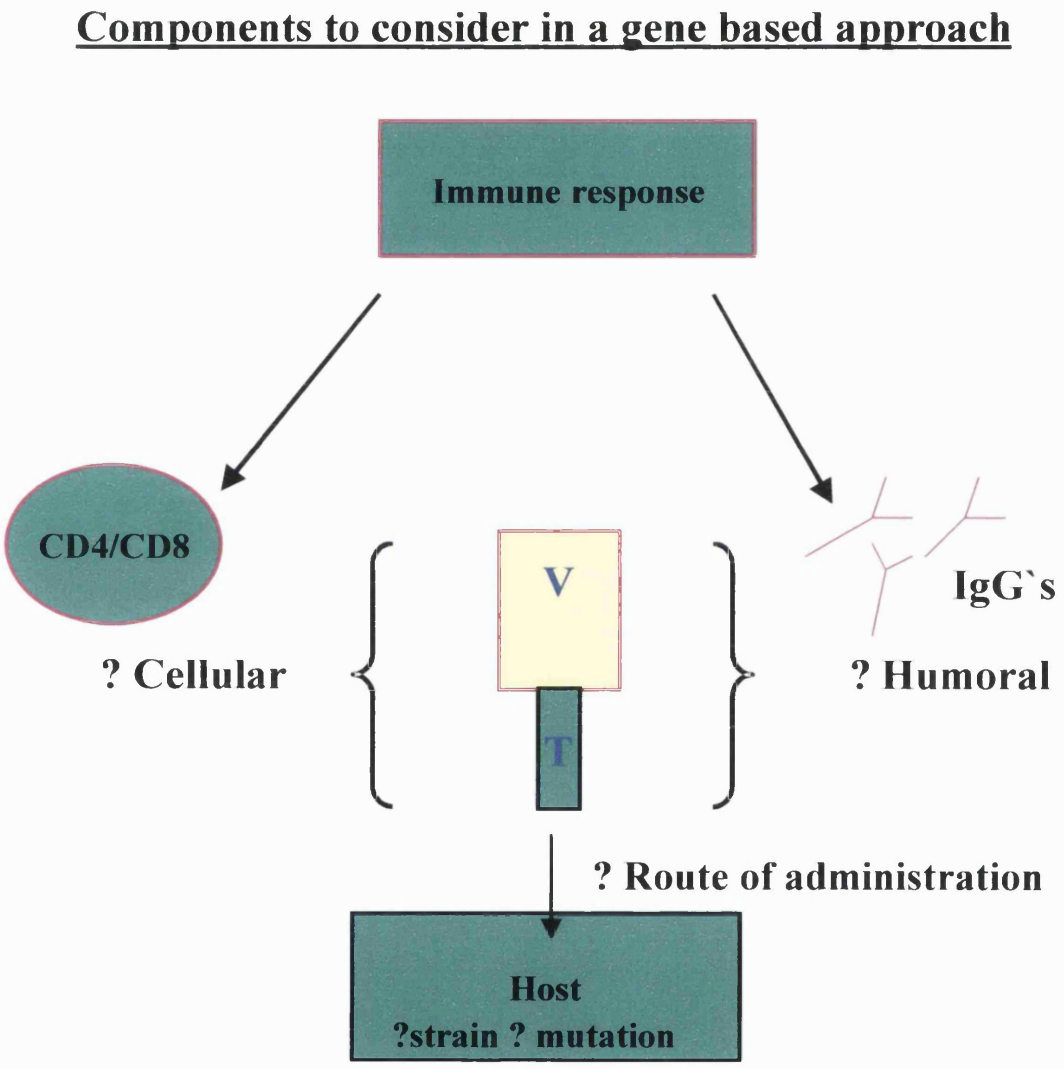
Figure 10 *Immune response and Gene Expression*



The mediators of this response may be cellular (CD4, CD8) or humoral (immunoglobulins), and there are several components of the treatment process where an immune response may occur. The components may broadly be divided into ***external*** factors (characteristics of the vector and the transgene) and ***internal*** factors

(characteristics of the host to receive gene therapy). The immune response may occur to one, both (or more) of the components as illustrated in *Figure 11.*

Figure 11 *Immunological components in a Gene Based Approach*



(Key: T = Transgene, V= Vector)

2.2 Immunological Components of a Gene Based Approach which may limit success

There are several points at which the immune response may limit overall efficacy of a gene transfer approach. The immune response may occur to endogenous host factors or exogenous factors, as outlined in the figure above (*Figure 11*).

2.2.1 External Factors

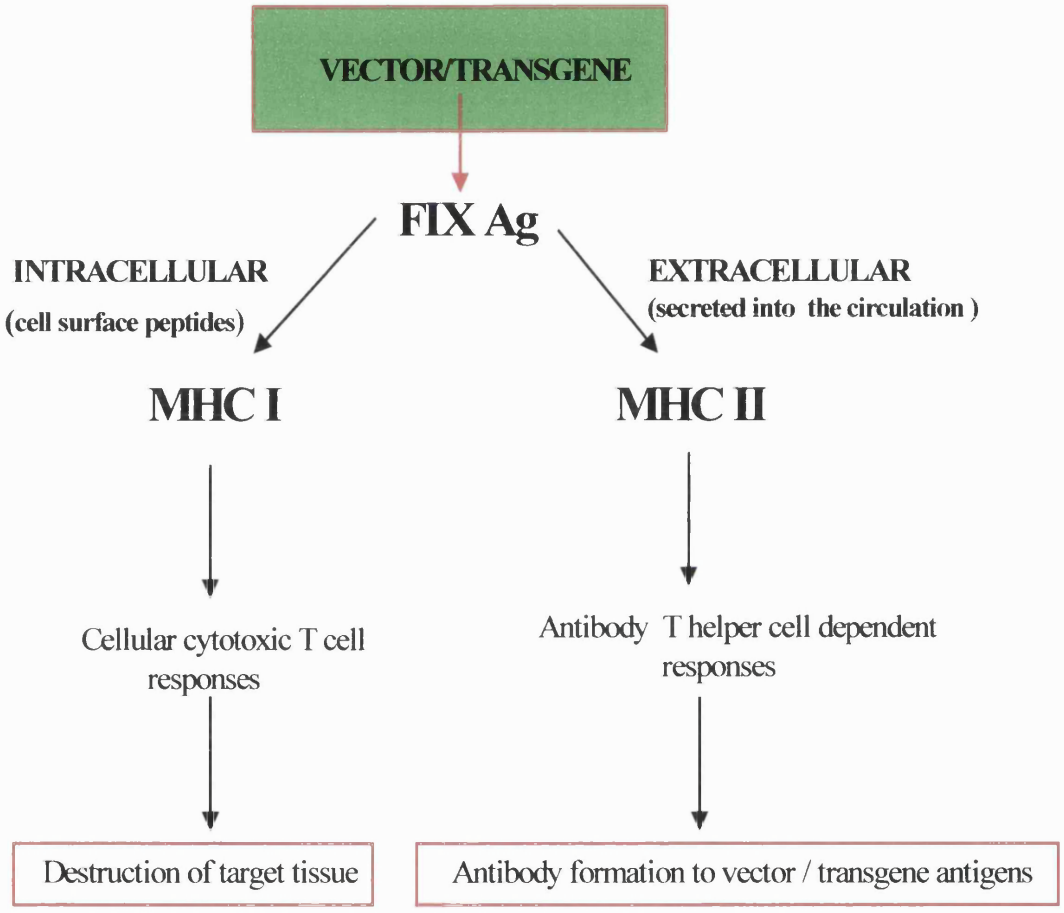
a) Transgene Selection

With the introduction of any new form of therapy for haemophilia it is important to anticipate the consequences of a possible immune response to the transgene product of the factor IX gene. It is known from previous introductions of new forms of therapy, that this is likely to occur. In the conventional protein based approach, intravenous infusion of either recombinant or plasma derived clotting factor concentrate may generate an inhibitory antibody response mediated by an MHC II pathway. This results from the activation of CD4 positive cells and their subsequent cognate interaction with B cells (Singer *et al.*, 1996; Reding *et al.*, 1999), resulting in antibody production. Therefore a humoral antibody mediated response may result from the transgene product human factor IX once it is secreted from the target cells into the circulation.

However, in a gene based approach an added layer of complexity is introduced, since the protein is manufactured intracellularly. The resultant peptides of the produced protein will be presented to the effector immune system on the surface of the target cell via a MHC I class pathway. This could theoretically lead to a chance of eliciting a cytotoxic T cell response (CTL) response to the peptide fragments presented on the cell surface and lead to elimination of the target cells manufacturing the novel proteins. This is in addition to the immune response, which may also occur to the proteins once they are secreted from the target cell, which is mediated via MHC class II pathways. Thus, in any gene therapy approach for haemophilia, one has to consider the additional interaction of an MHC class I response. *Figure 12* illustrates the

pathways involved in MHC presentation, with the differences between the protein based and gene based approach emphasised.

Figure 12 *Gene versus Protein based*



Intrinsic properties of the transgene

The properties of the transgene itself are also crucial to the overall outcome. With reference to haemophilia, the transgene product (factor VIII or IX) needs to be ***secretable***. However, most gene transfer studies addressing the possible problem of a CTL response to the transgene product have used the β galactosidase gene as a reporter gene. This gene is bacterially derived and non-secretable, and is clearly not representative of what may happen to a species-specific homologous transgene, which is required for use in haemophilia. Prior to undertaking this thesis there was no published work examining a possible CTL response to a secretable protein.

Fortunately, the recent availability of the relevant homologous transgenes and haemophilic animal models allows these questions to be addressed.

b) Vector selection

The vector used for gene transfer may influence the immune response to the vector itself and the transgene that it encodes. If the vector is highly immunogenic, it may trigger an immune response to the vector and possibly, even the transgene it encodes. The immune response may once again be cellular or humoral. In a humoral driven response the antibodies generated may lead to enhanced clearance of a vector, and more importantly, may preclude vector readministration.

2.2.2 Internal Host Factors

In the evaluation of an immune response to a new form of therapy for haemophilia, host cell factors play a critical role in predicting whether or not an immune response occurs. This is well demonstrated in the pathogenesis of inhibitor formation, where it is clear from numerous studies in replacement therapy of haemophilia, that underlying mutation plays a role in inhibitor formation. For these reasons stated above, there is more complexity in the gene-based approach.

2.3 Primary Objectives and Experimental Approaches Adopted

The primary objective of this thesis was to define the role of an immune response in a gene based approach, by characterising its occurrence both at a cellular and humoral level. In particular, a specific aim was to ascertain whether the process was T cell dependent or, independent.

Experiments were designed to investigate the humoral immune response in a muscle directed approach in both normal immune competent animals and in disease models of haemophilia B. The influence of vector selection was also examined and the effect it may have had on the resulting immune response. Additionally, for a gene-based approach to treating haemophilia, there also exists the potential for a cellular based response, as the protein is produced intracellularly and therefore affords the

opportunity of a CTL response to the produced protein. A second objective therefore, was to examine whether a CTL response could be elicited to the secretable transgene product human factor IX.

In the conventional replacement therapy one of the likely factors, which is associated with the development of an inhibitory immune response, is the nature of the underlying mutation causing haemophilia. A further objective was to examine the role of inhibitor formation in an animal model where the disease was caused by a large gene deletion, and one where the disease results from a missense mutation.

In designing these experiments, other factors considered were the route of gene delivery and its relationship to the likelihood of an immune response, and in particular whether certain routes of administration lead to a more tolerant state. In studying this, possible mechanisms were explored to explain the results observed. A final aim of the thesis was to explore ways of overcoming the immune response if it occurred, by means of immunomodulatory drug or antibody therapies specifically designed to block activation pathways of the immune system. In summary therefore, the thesis had three main objectives to:

1) To define the role and outcome of the immune response to a gene-based approach for the treatment of haemophilia B. The main emphasis, was to study humoral and cellular responses to the human transgene product factor IX in the context of AAV directed muscle based gene transfer.

2) To define factors both exogenous and endogenous, to the process, which may affect outcome of the response.

3) To try to overcome any possible response by the design of immunomodulatory strategies.

The thesis does not set out, or attempt to cover, all the aspects of the immune response, but primarily the work was performed to define some of these responses, which were not known about at the outset of these studies.

CHAPTER 3

MATERIALS AND METHODS

This chapter is divided into three primary sections, according to whether the techniques fell into one of three categories listed.

- **Clotting based assays**
- **Immunological assays**
- **Molecular assays**

3.1 Clotting Based Assays: Murine Assays

3.1.1 Modified APTT

The assay was performed by mixing 50µl of murine test plasma and citrate buffer with 50µl FIX deficient plasma (Organon Technika, Durham, NC) and incubating with 50µl APTT reagent (Organon Techninka) for 3 minutes at 37°C. 50µl of 25mM $CaCl_2$ was then added and the time to clot formation was measured with a fibrometer (Fibrosystem Cockeysville MD)(Kung *et al.*, 1998).

3.1.2 Bethesda Assay

The test plasma was taken and serial dilutions were made in Factor IX deficient plasma. 50µl of test plasma was incubated with 50µl normal mouse pooled plasma for 2 hours at 37°C. Following the incubation, 50µl of the mixture was added to 50µl of factor IX deficient plasma and incubated with 50µl APTT reagent. The APTT was then recorded and the residual factor IX was calculated by using a standard curve, which was generated from normal mouse plasma. The Bethesda titre was then calculated and adjusted according to the dilution of the test plasma.

3.2 Canine Assays

3.2.1 Whole blood clotting time

The whole blood clotting time was determined as follows: 2ml of freshly drawn venous blood was added to separate 5ml red top tubes. The first tube was inverted

every 2 minutes and inspected for clotting. When a solid clot was observed in the first tube, the second tube was inverted and inspected at 1-minute intervals. The time at which a solid clot formed in the second tube was recorded as the WBCT.

3.2.2 APTT clotting time

Activated partial thromboplastin time (APTT) of plasma samples were measured with a fibrometer (Fibrosystem, BBL, Cockeysville, Maryland). 50µl plasma samples and APTT reagent (Organon Teknika, Durham, North Carolina) were mixed each and incubated at 37°C for 3 minutes, before addition of 50µl of 25mM $CaCl_2$ and measurement of the clotting time with the fibrometer.

3.3 Immunological Assays

Murine

3.3.1 Factor IX Ag elisa for mouse

For coating 6.5µl, of A300 rabbit anti hFIX (Dako corp, US) was diluted into 6.5ml of coating buffer and 50µl of this was added to each well, using a multichannel pipetter, The plate was left to incubate overnight at 4°C. The plate was then washed 4 times using washing buffer (1xPBS w/Tween). 200 µl of diluting buffer was then added to each well, using multichannel pipetter as the blocking stage and the plate incubated at room temperure for 1 hour.

The standards were thawed: (Factor IX: 200, 100, 50, 25, 12.5, 6 and 3 ng/ml) and placed on ice. The serum samples tested (or media collected from cell culture) were then thawed. The plate was washed 4 times using washing buffer (1 x PBS w/ Tween), tapping the plate on the last wash onto a paper towel to empty the wells. The standards (100µl) were added to each well in the first 2 columns (in duplicate). A blank was added (diluting buffer) to the last row. 100µl of each sample was added in duplicate in each well. The plate was incubated at 37°C for 2 hours, and then washed.

The detection stage was carried out by diluting 4.5µl HPO-conjugated goat anti-human factor IX into 10.5 ml diluting buffer and adding 100 µl to each well. The plate was incubated at 37°C for 2 hours and then washed. The substrate was made up using 12-mg O-phenylenediamine + 12 ml 0.01M Na-citrate, and placed on ice. The substrate was then activated with 2.5 µl of hydrogen peroxide and a 100 µl of substrate was added to each well, incubated at room temperature for ~10-15 minutes, before reading recording the optical density at 450 nm.

3.3.2 Subclass specific Elisa (for anti Human IgG subclasses in mouse plasma)

96 well Elisa plates were coated with human derived plasma factor IX (1 µg /ml, mononine, Armour Pharmaceutical Co, Kankaee, IL) overnight at 4° C. The following morning the plates were washed and serum from the test mice was added at a dilution of 1/32. Following a 2-hour incubation at 37°C, the plates were washed and anti human factor IX was detected with horseradish peroxidase conjugated antibodies mouse monoclonal antibodies (1:2000) specific for mouse IgG1, IgG2a (cross reacting with IgG2c), IgG2b, IgG3 or IgGM (Boehringer- Mannheim, Indianapolis, IN). Following a 2-hour incubation the plates were washed. The detection was carried out using a peroxidase enzyme system, with the substrate reagent o-phenylene diamine (12 mg o-phenylenediamine + 12 ml 0.01M Na-citrate). The OD was read at 450nm.

Antibody titres in µg IgG /ml were determined by standard curves for wells coated with serially diluted purified murine IgG proteins (IgG1, IgG2a, or IgG2b, starting at 16 µg IgG /ml; Sigma, St Louis, MO).

3.3.3 Subclass specific Elisa to measure antibody subclasses to murine Factor IX

To perform this Elisa, wells were coated with mouse Factor IX (1 µg/ml), blocked, and then incubated with mouse plasma (1:16). Parallel wells were coated with two fold dilutions of purified immunoglobulins (Sigma, St Louis, MO) starting at 100ng/ml for generation of the standard curve. Secondary antibodies were specific for mouse IgM, IgG1, IgG2a, IgG2b, and IgG3 (Horseradish peroxidase label, Boeringer

Mannheim). Optical density was read at 450 nm after incubation with substrate o-phenylene diamine (12 mg o-phenylenediamine + 12 ml 0.01M Na-citrate).

3.3.4 Cytokine Elisa for Murine Cytokine assays

These assays were performed as per manufacturers instructions (Pharmingen, US).

Coating – Capture antibody:

The purified anti-cytokine capture antibody was diluted to 1-4 μg/ml in coating buffer (CB) and 50μl added into each well (repeat by mixing and removing with 50 μl to ensure uniform coating. The plate was subsequently incubated at +4°C overnight (in the cold room). The dilutions for each cytokine capture antibody are shown in the table below.

Cytokine dilutions for capture antibody (purified rat anti-mouse monoclonal antibody):

	Final Conc^{n}:	Stock:	Dilution:	For 2ml CB:
IL-2	2 μg/ml	100 μg/ml	1:50	40 μl
IL-4	2 μg/ml	500 μg/ml	1:250	8 μl
IL-10	4 μg/ml	200 μg/ml	1:50	40 μl
IFNγ	4 μg/ml	1000 μg/ml	1:250	8 μl

The plate was removed from the cold room, and washed 4 times. Finally 200 μl of diluting buffer was added to each well and incubated for 1 hour at room temperature prior to sample loading.

Loading samples and cytokine standards

Prior to loading the samples, the cytokine standards were prepared as follows.

IFNγ /IL-2

To make up these standards, serial dilutions were made from a stock solution of 1 μg/ml, starting with a highest concentration of 5000 pg/ml (1:200 dilution) and then performing doubling dilutions down to a concentration of 39 pg/ml.

IL-4, IL-10

To make up these standards serial dilutions were made from a stock solution of 50 μg/ml starting with a highest concentration of 2000 pg/ml (1:25000 dilution) and then performing doubling dilutions down to a concentration of 15 pg/ml. Before adding either the standards or test samples, the plates were washed three times. Then the standards and test samples are added at 100 μl per well in duplicate and left to incubate room temperature for 2 hours. The plate was then washed 4 times and the detection antibody added as follows:

Detection-antibody

The biotinylated anti-cytokine detecting antibody was diluted to 0.25-2.0 μg/ml in DB (Diluting buffer, PBS, 0.05% Tween, 6% Bovine serum albumin):
100μl of the antibody was added to each well and the sample allowed to incubate for 1 hour at room temperature. The plate was washed 6 times.

	Final conc:	Stock:	Dilution:	For 2ml CB:
IL-2	1 μg/ml	100 μg/ml	1:100	20 μl
IL-4	0.5 μg/ml	100 μg/ml	1:200	10 μl
IL-10	1 μg/ml	500 μg/ml	1:500	4 μl
IFNγ	1 μg/ml	100 μg/ml	1:100	20 μl

Avidin-Peroxidase

The avidin- or streptavidin-HRP conjugated enzyme was diluted to the manufacturers optimal concentration in DB (1:1000), and 100 μl added to each well and incubated at room temperature for 30 minutes. The plate was then washed 8 times prior to the detection stage.

Substrate and Detection

The substrate was made with 12 mg o-phenylenediamine + 12 ml 0.01M Na-citrate and placed on ice. The substrate was then activated with 2.5 μl of hydrogen peroxide

and 100 µl of substrate added to each well, and incubated at room temperature, looking for the colour change (~ 10-15 min). The wells optical density was then read on a photodetector at 450 nm.

3.3.5 Western blot

The western blot was performed by electrophoresing 1µg of murine factor protein onto a 7.5% polyacrylamide gel, and transferring it onto a nitrocellulose membrane using an electroblot system (Biorad). Subsequently, the test plasma, at a dilution of 1/500 was added and then incubated for 1 hour. A secondary antibody, anti-mouse IgG conjugated to horseradish peroxidase (Boehringer Mannheim, Indianapolis, IN) was used at 1:1000 dilution and was allowed to incubate for 1 hour. The strips were subsequently washed 6 times and then substrate was added for 5 minutes before detection by autoradiography.

3.4 Murine Proliferation Assays

The technique used for the murine lymphocyte proliferation assay was as follows :

1. Dissection of tissues

The mice were euthanised, and then prepared for removal of the spleen and draining lymph nodes (popliteal and inguinal). The spleen was dissected out and placed in complete medium (Iscove's MDM w/ 25mM Hepes, L-glutamine, P/S, 5-10% FBS, pyruvate and $2x10^{-5}$ M 2-ME [180 µl $5.5x10^{-2}$ M 2-ME into 500 ml Iscove's makes $2x10^{-5}$ M] using a 6-well plate, transferring on ice until the beginning of the proliferation procedure. Similarly, the draining lymph nodes (Inguinal and popliteal) were dissected away from the injected animals and placed in completing medium and allowed to sit on ice, and the fat cells allowed to collect at the top of the tube. The cells were subsequently removed and the lymph node cells were kept on ice

2. *Preparation of cell fractions*

The tissues were homogenised using a fine wire mesh and sterilised by burning in C_2H_5OH. The sterilised mesh was placed in a 6-well plate containing 3 mls of wash medium (Iscove's MDM w/ 25 mM Hepes, L-glutamine, P/S and 5% FBS). The tissue was placed between the two layers of the mesh and homogenised using a 3-ml sterile syringe plunger (max 2 spleens/mesh) to crush the tissue. The cell suspension was transferred into a 15-ml conical flask and repeated once again with fresh wash medium to collect the remaining cells, equalising the tubes with wash medium prior to centrifuging. The samples were transferred on ice.

The samples were spun at 500 g at +4 °C for 5 min. The supernatant was removed. The side of the conical flask was then tapped to displace the pellet, and 6 ml AKC (lysing) buffer was added to the dislodged pellet. The lymph node and spleen cells were then combined and left on ice for 2-3 minutes, to lyse the red cell component away from the harvested tissues. The samples were respun and the fractions were washed with 5-mls of wash medium, resuspended and centrifuged. Finally, the supernatant was removed and the cells were resuspended in 5 ml of complete medium prior to cell count being performed.

3. *Cell count*

A cell count was performed using a 1:1 dilution of cells in Tryptan blue (20µl dye + 20µl cell suspension) and then placing 20µl of the mixture into the counting chamber - haematocytometer. Using a counting microscope, a cell count was performed.

4. *Plating out cells – proliferation plate (96 well)*

For the cell proliferation assay, Concanavalin A (10ug/ml) was used as the positive control (Concanavalin A is a proliferating agent, which works by crosslinking B- and T cell receptors).

The test stimulator for the proliferation assay was human factor IX. The factor IX has to be added to the effector cells in serial dilutions. The factor IX was prepared by

making doubling dilutions starting from the highest concentration of 20μg/ml down to 0.3μg/ml. For a negative control, 100μl mock medium was used i.e. complete medium without any added antigen (last row of the plate). The cells were added to the proliferating medium at a density of 100 μl (which equalled a density of $5x10^5$ cells/well). The final volume per well was therefore 200 μl. (All mediums used for the plating out stage were in complete medium – i.e medium containing 2ME, 5% FBS, Sodium pyruvate, P/S, Icoves). The cells were then left to proliferate with the factor IX and Concanavalin A in the 5% CO_2 incubatorat 37°C, for five days, prior to cell labelling with tritiated thymidine.

5. *Cell Labelling*

After day 5 of the proliferation, ^{3} H Thymidine was added to each well of the culture. 1 μCi is added to each well in a volume of 20μl using the radioactive multipipetter. The cells were pulsed overnight and harvested onto glass fibre filters using a 96 channel cell harvester (Nunc Gibco), washing 4 times with distilled water. Filters were air-dried and the ^{3}H Thymidine content was determined by liquid scintillation counting.

6. *Cytokine release assay*

The cytokine release assay was carried out in a 24 well plate. First, the stimulating antigen was added to the plate in complete medium by starting with the highest concentration of 20μg /ml human factor IX (Mononine, Alpha therapeutics, US) and making doubling dilutions down to 1.2μg/ml. Concanavalin A (con A) was used for the positive control at a concentration of 6μg/ml. The negative control was mock medium, i.e. complete medium without any added antigen (last row of the plate). Finally, the effector cells were added at a density of $5x10^6$ cells per ml. The final volume per well was therefore 1.5ml. The cells were incubated at +37°C in a cell culture incubator with CO_2 at 10 % for 72-120 hours. The supernatants were harvested and stored at -80°C until analysis via cytokine Elisa as described above.

3.5 Canine Assays

3.5.1 Canine Factor IX Elisa

Canine factor IX Ag levels were determined by Elisa using a mouse monoclonal anti human factor IX (Boehringer) as the capture antibody (this antibody cross-reacts with canine factor IX). Plates were coated with 1.5μg/ml antibody per ml coating buffer. The detection immunocomplex was a rabbit anti canine factor IX (Affinity biological, Hamilton Ontario, Canada) at a 1:1400 dilution using a stock solution of 10mg/ml (total IgG) and a horse-radish peroxidase conjugated swine anti rabbit immunoglobulin (with no cross reactivity with canine or murine Immunoglobulin, 1:2000 dilution, Dako, Carpinteria, California). Purified plasma derived canine factor IX (Enzyme Research laboratories, South Bend, Indiana) was used as the standard. The lower limit of detection of the assay was approximately 1.5μg/ml

Anti canine Ab assays

Antibodies against canine factor IX were detected either by Western Blot or Elisa.

3.5.2 Elisa

Wells of a microtitre plate were coated with purified canine factor IX protein (1ug/ml), blocked with 6% bovine serum albumin (BSA) in PBS, pH 7.4 solution, and dilute serum samples were added (1: 16 in BSA/PBS). Antigen antibody complexes were detected in horseradish peroxidase conjugated antibodies specific to canine IgM (1:1000), IgG1 (1:2000), IgG2 subclasses (1:2000) (Bethyl Laboratories, Montgomery, Texas). For the detection step, the substrate was made up as follows: 12 mg o-phenylenediamine + 12 ml 0.01M Na-citrate, place on ice. The substrate was activated with 2.5 μl of hydrogen peroxide. 100 μl of substrate was added to each well, and the incubation was carried out at room temperature for ~10-15 min. The optical density was read at 450nm. For the quantitation of results, wells were coated in parallel, using two fold serial dilutions of canine reference serum with known concentrations of immunoglobulins (Bethyl laboratories).

3.5.3 Western Blot anti cFIX Ab

Membrane strips with 1μg of purified canine factor IX were incubated (Enzyme Research laboratories, South Bend, Indiana) with canine test serum dilutes 1:200. Rabbit anti canine IgG (with a horse radish peroxidase label) diluted 1:1000 (Sigma) was used as the antibody for chemiluminescent detection.

3.6 Canine Proliferation assay

This assay was designed to look for the presence of proliferation in the CD4 cellular fractions to cFIX antigen in a canine model of haemophilia injected with intramuscular Adeno- associated vectors (*see Chapter 8*). I wish to acknowledge the helpful comments of Professor P Felsburg (University of Pennsylvania Veterinary School) and Dr J Brubaker (Wistar Institute) in the setting up of this assay.

Method

Blood samples (15 ml) were collected in preservative-free heparin as the anticoagulant and maintained at room temperature prior to isolation of lymphocytes. Blood samples were separated by centrifugation in HEPES-buffered Hanks Balanced Salt Solution followed by a ficoll gradient (Sigma Hypaque Ficoll 1.077, Sigma Chemical Company, St. Louis, MO) to remove platelets and red blood cells. Banded peripheral blood mononuclear cells (PBMCs) were washed twice in HEPES-buffered Iscove's medium (GIBCO BRL, 25 mM HEPES) and remaining red blood cells were removed by lysis. PBMCs were cultured in lymphocyte proliferation medium (Iscove's medium including glutamine, 1 mM sodium pyruvate, $5x10^{-5}$ M 2-mercaptoethanol, 5% fetal bovine serum, and antibiotics) in microtiter plates at $5x10^5$ cells/well.

After five days of culture (37°C, 10% CO_2 in air) in the absence (mock) or presence of canine factor IX (two-fold serial dilutions of purified plasma-derived protein, Enzyme Research Laboratories, South Bend, ID, starting with 20 μg cFIX/ml medium), or presence of the mitogen concanavalin A (1 μg/ml), cells were pulsed with ^{3}H-thymidine for 16 hours. Proliferation for each concentration of canine factor

IX was assayed in quadruplicate. The cells were then harvested the following morning using a Tomotec harvester (Harvester 96 Mach III).

The cells were harvested onto glass filter mats (Wallac 1450-421). Once the mats had dried, the mat was placed in bag containing Scinti fluid and the degree of triated thymidine uptake was measured using a Beta lux cell scintillation counter (Nunc). The stimulation index was calculated as the ratio of the average proliferation of canine IX-stimulated cells vs. mock-stimulated cells. A stimulation index >2 was recorded as a positive result.

3.7 Histological analysis

3.7.1 Murine

Haematoxylin and eosin staining

Frozen sections were rehydrated by incubating in decreasing concentrations of alcohol (100-100-95-80-75%), before placing them in de-ionised water. They were then placed in Elrich`s haematoxylin for 10 minutes. The slides were then rinsed in running tap water for 2-5 minutes and transferred in acid–alcohol (1% ethanoic acid, 70% ethanol) for 30 seconds. They were then rinsed again in running tap water and were transferred in eosin for 1-5 minutes. They were then placed in de-ionised water and incubated in a series of alcohols (70-80-95-100-105%) before placing them in histoclear for 5 minutes. Following this, the slides were coverslipped-using DePex (BDH).

Immunofluorescence staining for CD8 deposition in mouse muscle

Cryosections were fixed in acetone, air-dried and subsequently blocked in 1% serum/ 1x PBS for 10 min. FITC-conjugated anti-CD8 (Pharmingen, San Diego, CA) was applied at a 1:25 dilution in 1% serum/1x PBS and incubated for 1 hour. Cryosections of draining lymph nodes of the injected muscle were also stained with peanut agglutinin (PNA)-biotin (Caamono, *et al.*, 1998).

3.7.2 Canine

Muscle biopsies (100-200mg of tissue) of injected and non-injected tissue were excised with a sterile scalpel from dogs sedated by general anaesthesia with coverage with normal canine plasma that began before the procedure. Excised tissue was snap frozen and cryosections were prepared (6μm) and stained with haematoxylin and eosin. To look for the presence of canine factor antigen, a primary antibody dilute 1:100 (Affinity Biologicals, Hamilton Ontario) was applied and a secondary antibody FITC –conjugated swine anti rabbit immunoglobulin diluted 1:30 (Dako, Carpinteria, California) was applied.

3.8 Generic Molecular Techniques

In order to start the construction of a vector for any gene transfer protocol, a number of molecular techniques are required in the cloning steps for the production of suitable plasmid vectors, either for use as vectors themselves or for use later in viral vector preparations.

Techniques acquired for Cloning of Vectors

3.8.1 Restriction Digests

These were required to manipulate various cDNA sequences from the provided plasmids i.e. from other investigators and the ATCC. The plasmid map was studied and the appropriate restriction sites identified. Then, under conditions as dictated by the individual restriction endonucleases, restriction digests were carried out. The endonucleases were supplied by Boeringher Mannheim, GIBCO, or Promega. Reactions were typically carried out using 10U of enzyme in a 20μl reaction with the relevant buffer. In any one reaction usually about 1-2μg of DNA was digested.

3.8.2 Ligation Reactions

Reactions were set up in 20μl volumes containing approximately 10 ng of vector and 50-200ng of insert in 1x ligation buffer (10 mM rATP, 1 M Tris –HCL pH 7.4, 1 M $MgCl_2$ and 0.3 M DTT). One μl of T4 DNA ligase (IU Boeringer) was added, the

reaction mixture made up to 20µl with sterile distilled water (SDW) and the reaction was carried out overnightat 16° C.

3.8.3 Alkaline phosphatase reaction

This reaction was carried out to dephosphorylate the vector in order to prevent re ligation of linearised DNA molecules during cloning procedures. Briefly, calf intestinal phosphatase (CIP) was used to remove the 5` terminal phosphate group of the digested DNA fragments. One µl of CIP was added to the restriction digest mixture, and incubated for 1 hour at 37°C. Then, 5mM EDTA pH 8 was added and the mixture heated to 65°C for 20 minute to inactivate the CIP. The DNA was then phenol chloroform extracted. The aqueous phase containing the DNA was subsequently ethanol precipitated and resupended in TE 20µl.

3.8.4 Agarose gel electrophoresis

Agarose gel of the various required percentages were made by dissolving Agarose in either 1X Tris–borate (TBE) buffer (90mM Tris pH 8, 90mM orthoboric acid, 2.5mM EDTA) or 1x Tris acetate (TAE) buffer (x 50 buffer contains 242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8.0) made up to 1 litre with de-ionised water). To visualise DNA, ethidium bromide was added at a final concentration of 0.5 µg/ml. The gel was the then poured into a running tray and a comb was added to the tray, which was then removed when wells formed prior to the DNA samples being added. Once the tape and comb were removed the, appropriate buffer x1 was added (depending upon what the gel was made from), 10 µl of sample was mixed with 5 µl of x 6 loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 40%, (w/v) sucrose in water). An appropriate molecular weight marker was used to determine the size of the DNA fragments. Typically, 0.7% w/v agarose was used for separation of fragment sizes between 0.8Kb-12Kb, whereas 0.5% w/v agarose separated between 0.1Kb-30Kb and 1.5% w/v between 0.2Kb-3Kb. The gels were run at 45-55mA and the negatively charged DNA then migrated towards the anode. The DNA was visualised using a UV transilluminator and photographed with a Polaroid black and white camera.

3.8.5 Bacterial transformations

A 100µl of competent cells (Epicurian Coli SURE 2 supercompetent Cells, Stratagene) were taken and thawed on ice in an eppendorf tube. In a volume of 50 µl, a maximum amount of 50 ng of DNA taken and mixed with the competent cells and left on ice for a maximum of 30 minutes. In a 42°C water bath, the cells were then heated shocked for 90seconds and then placed on ice for 2 minutes. 900 µl of LB SoC medium (50mls LB medium, 0.5mls 1M $MgSo_4$, 0.1mls 20% Glucose) was added to the cells and the mixture was incubated for 1 hour at 37 ° C. The cells were then spun down and streaked out over LB/Ampicillin (Amp) plates. LB/Amp plates were made up from a autoclaved mixture of: 500ml de-ionised water, 5g tryptone, 2.5g yeast extract, 5g NaCl, 7.5g Agar. The mixture was allowed to cool, and ampicillin was added at a final concentration of 50µg/ml. The mixture was the added to 90 mm petri dishes and allowed to set. The streaked out bacteria were the incubated at 37°C overnight.

3.8.6 Minipreps: (Required for Colony Screens of cloning vectors)

The petri dishes were removed from the incubator and a discrete colony was picked off the plate using a sterile toothpick and aseptic technique, and inoculated into 4mls LB/Amp. The mixture was placed in a shaker at 225 rpm overnight at 37°C. 1.5 mls of the mixture was centrifuged and the supernatant removed. The pellet was resuspended into 100µl of solution I (43.75ml H_2O, 1.25ml Tris Stock 1M, 5ml 100mM EDTA, 0.45g glucose) and resuspended. Then 200 µl of solution II (100µl 10% SDS, 20µl 10M NaOH, 880µl H_2O) was added to the mixture, inverting several times.

150µl of solution III (30 ml 5M Potaasium acetate, 5.75ml Glacial acetic acid, 14.25ml H_2O) was added, and the solution was shaken vigorously. The mixture was centrifuged in a microfuge (1300rpm) for 2 minutes. The supernatant was removed to a tube containing 400µl phenol/chloroform. The tube was shaken vigorously for 1 minute and then centrifuged again for 2 minutes. The supernatant was removed and added to a tube containing 200 µl of chloroform and centrifuged for 2 minutes. The supernatant was removed and added to 800µl of ethanol 100%, vortexed and

centrifuged for 2 minutes. The ethanol was removed and the pellet washed in 70% ethanol. The ethanol was removed and the pellet left to air dry and finally resupended in TE.

3.8.7 Maxiprep (for large scale production of DNA)

Once the DNA was deemed to be correct, a sample was then grown up by maxipreping according to the Quiagen protocol. 150 mls of grown culture was taken and spun down to create a bacterial pellet, which was resupended in 10mls of buffer P1 (43.75mls H_2O, 1.25mls Tris Stock 1M, 5mls 100mM EDTA, 0.45g glucose). The bacteria were completely redissolved and 10mls of buffer P2 (100µl 10% SDS, 20µl 10M NaOH, 880µl H_2O) were added and incubated at room temperature for 5 minutes. The solution was mixed thoroughly by inverting the tube 5-6 times. Then 10 mls of buffer P3 (30 ml 5M Potaasium acetate, 5.75ml Glacial acetic acid, 14.25ml H_2O) was added and the solution mixed by inverting 5-6 times. The solution was then incubated on ice for 20 minutes. After incubation, the sample was mixed again before centrifugation was carried out at 15000 RPM for 30 minutes at 4°C.

After centrifugation the supernatant was removed promptly and applied over a Qiagen tip 500 which had been equilibrated by applying 10 mls of buffer QBT (750mM NaCl, 50 mM MOPS, pH 7.0, 15% isopropranol, 0.15% Triton X-100. The supernatant was applied and allowed to drain through the column by gravity flow. Once drained, the column was washed twice with 30 mls of buffer QC (1.0M NaCl, 50mM MOPS, pH 7.0, 15% isopropranol). The DNA was then eluted off the column with 15 mls of buffer QF (1.25M NaCl, 50mM Tris Cl, pH 8.5, 15% isopropranol). The DNA was then precipitated with 0.7 volumes of isopropanol previously equilibrated to room temperature. The mixture was centrifuged immediately at >15000g at 4°C for 30 minutes and the supernatant carefully removed. The DNA was washed in 15 mls of cold 70 % ethanol and redissolved in TE. The yield of DNA was calculated by measuring the DNA concentration on a UV spectrophotometer at 260nm.

3.8.8 Transfections

For the transfection, the cationic lipid lipofectamine was used. Lipofectamine interacts with DNA to form a lipid DNA complex. The resulting lipid – DNA complex fuses with the tissue culture cells under test and results in uptake and expression of the DNA to the target cells.

3.9 Specific Molecular Assays

PCR reactions

The polymerase chain reaction (PCR) was used for a variety of purposes: 1) To detect the presence or absence of specific DNA sequences, using DNA extracted from cells (murine, canine) or tissues. 2) To amplify specific sequences for further downstream manipulations (i.e. inserts for cloning), engineering convenient restriction sites within the primers. 3) For use in RT-PCR reactions on RNA extracted from various tissues.

For routine PCR reactions, the Taq DNA polymerase was used. Preliminary reactions were carried out to optimise reaction conditions, with variables such as primer annealing temperature, extension time and Mg^{2+} concentration. In all reactions, a control was included as a negative control to detect contamination if present. Additionally, a control was included that was not known to contain the target DNA sequence or that contained an amplifiable sequence of different length to the primary target sequence. PCR products were visualised either by agarose or polyacrylamide gel electrophoresis. Routine PCR reactions of 20 µL were set up in 1x Taq reaction buffer (Promega), 0.2mM dNTP's, 2-10 pmol of each primer (forward and reverse), 1-2.5 mM $MgCl_2$, DNA template (2µl) and 2.5U of Taq polymerase (0.5µl, Promega). The DNA template varied from 10 ng (plasmids) to 100ng- 400ng (DNA from tissues). Mineral oil (Sigma) was placed on top of the mix and the tubes were placed on a Biometra Trioblock PCR machine. Generally, 30-35 cycles were carried out, with an initial 95°C template denaturation step (1 min), 55-72° C primer annealing (1 minute) and 72 extension (1-3 minutes) .The reactions were placed on ice or at 4°C.

3.91 PCR Analysis of murine injected muscle

In three animals, at two months post injection, a RT-PCR reaction was performed on extracted muscle from the injected legs to look for the presence of factor IX cDNA. Total RNA was isolated from muscle using Trizol reagent (Gibco/BRL). RNA 1μg was reverse transcribed using a kit from Gibco/ BRL. PCR primers specific for murine factor IX cDNA were used to amplify 434 bp of the mouse factor IX transcript using one fifth of the cDNA product as the template (annealing temperature of 54°C, 35 amplification cycles). The primers used were as follows:

Forward primer 5'- GATTGTAACTGCTGCCCACTGTCT-3'
Reverse primer 5'- AATCTTTGCCTCCTTCCGGGTAGC-3'

No amplification product was obtained from uninjected muscle that was reverse transcribed.

3.92 RT- PCR protocol for Isolation of Canine cytokines from peripheral blood mononuclear cells

An assay was devised to measure the intracellular cell cytokine levels via an RT-PCR assay, due to the lack of available reagents to measure canine cytokines levels in the cell supernatants. PBMCs were cultured in 24-well plates at $2x10^6$ cells/well, and stimulated with canine factor IX (10 μg/ml) for 4 days. Subsequently, cells were harvested, and total RNA was isolated using the Trizol reagent kit from Gibco/BRL. RNA was reverse transcribed (1 μg/sample) using the Superscript kit from Gibco/BRL. 1/10th of the cDNA product served as a template for PCR amplification with "ready-to-go" PCR beads (Pharmacia Biotech, Piscataway, NJ).

PCR conditions were as follows: 5 minutes initial denaturation at 94°C, 40 cycles of 94°C (1 minute), 52°C for 1 minute (annealing for primer pairs for IFNγ or IL-2) or 60°C for 1 minute (IL-10 or β-actin primer pairs), and 72°C for 1 minute (extension), followed by a final extension step of 72°C for 10 minutes. PCR products were separated on an ethidium bromide stained 1.5% agarose gel.

PCR primer pairs for canine cytokines were as follows:

- **IL-2**: 5'-CATCGCACTGACGCTTGTA-3'(forward primer) 5'-CTTGTTTCAGATCCCTTTAGTTTC-3'(reverse primer) yielding a 345-bp PCR product
- **IL-10**: 5'-CAGCCGACACCAGAGCACCCTACT-3'(forward primer) 5'-AAATGCGCTCTTCACCTGCTCCAC-3'(reverse primer) yielding a 391-bp PCR product
- **IFN-γ:** 5'-CGGTGGGTCTCTTTTCGTAG-3'(forward primer) 5'-GCCTTGCGCTGGACCTG-3' (reverse primer) yielding a 255-bp PCR product
- **β-actin** 5'-GCGATGAGGCCCAGAGCAAGAGG-3' (forward primer) 5'-GTCCCGGCCAGCCAGGTCCAG-3'(reverse primer) yielding a 426-bp PCR product.

CHAPTER 4

VECTOR CONSTRUCTION AND PRODUCTION

Introduction

In any gene transfer protocol, the choice of vector to deliver the transgene product is crucial. Vectors all have individual properties, which may or may not make them attractive to any given protocol. As summarised earlier, the initial studies for expression of clotting factors in muscle were carried out using plasmid vectors as a vehicle for gene delivery.

For the process to be successful, efficacy must first be achieved *in vitro* before attempting to try those vectors *in vivo* to prove that the selected target tissues are capable of producing biologically active clotting factor genes. With respect to haemophilia, any tissue that can produce clotting factor genes is a potential target. Several authors have shown that it is possible to obtain clotting factor gene expression from a number of tissues: fibroblasts (Palmer *et al.*, 1989), myoblasts, (Yao *et al.*, 1991), endothelial cells Yao 1991. (Yao *et al.*, 1991) and keratinocytes (Gerrard *et al.*, 1993).

The early *in vitro* work in this chapter describes the strategy of using plasmid systems for assessment of the optimal expression cassette, prior to incorporation into a viral vector (AAV) system.

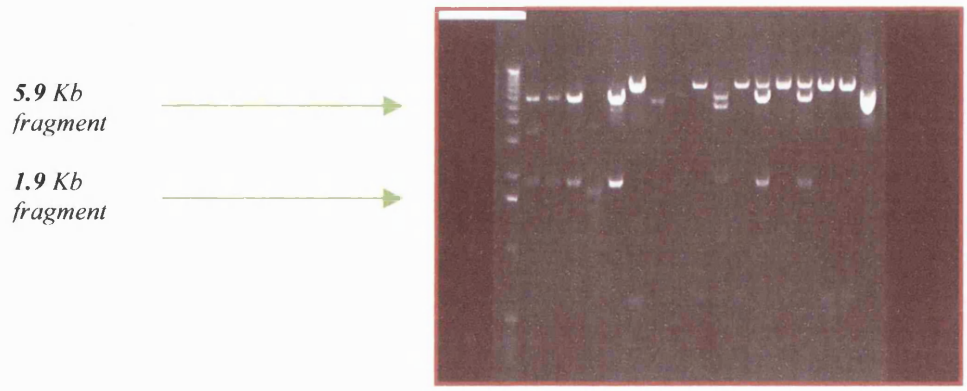
4.1 Plasmid Vectors

Initially because of lack of availability of the factor IX (FIX) gene, early work was performed using the factor VII (FVII) gene. The purpose of these experiments was to demonstrate proof of principle to show that the chosen target cells could secrete FVII *in vitro*. When FIX cDNA became available the studies were extended to test FIX cDNA expression cassettes *in vitro.* The majority of the experimental work performed in this thesis is using the factor IX gene either as a plasmid vector or in viral vector systems.

4.1.1 FVII Plasmid Vector: FVII/PCDNA3

A FVII vector was made by inserting the FVII cDNA into a commercially available expression vector PCDNA 3. Before these vectors were used *in vivo*, the vectors were transfected into a muscle cell line C2C12 and also other cell lines (Cos 7) to test expression capacity. The expression vector has a CMV promoter inserted which facilitates high-level transgene expression in muscle as a target tissue (Dai *et al.*, 1992a).

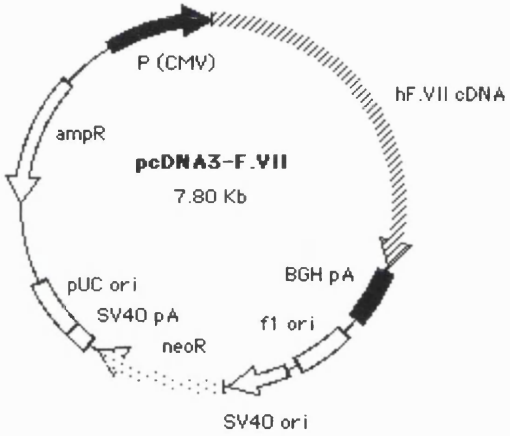
The FVII cDNA insert was cut away from its PUC 19 supply plasmid (via ECOR1 restriction digest) and extracted via a gene clean kit (Quiagen, MinElute Gel Extraction Protocol) from the agarose gel electrophoresis gel. The PCDNA3 plasmid was linearised with an ECOR1 digest and then dephosphorylated to avoid religation (as described in materials and methods). The vector and insert were then set up overnight in ligation mix at 16°C. The following day, the ligation mix was transformed into competent cells and then streaked onto agar plates overnight. The following day the colonies grown were picked from the plates and minipreps were screened to analyze the extracted DNA. The DNA was run out on a gel and the orientation of the construct was checked by a double restriction digest. The miniprep gel is shown below, which shows the correct 1.9-Kb and 5.9 Kb fragments in the correct orientation.



The colonies in the correct orientation were then madeup in large in quantities by growing up the vector using a maxiprep technique (Quiagen, Germany, Chapter 3, page 79). The DNA concentration produced was analysed by standard 260nm spectroscopic absorbance. The DNA was then stored and resuspended in glycerol buffer (15%) in small aliquots at -70°C.

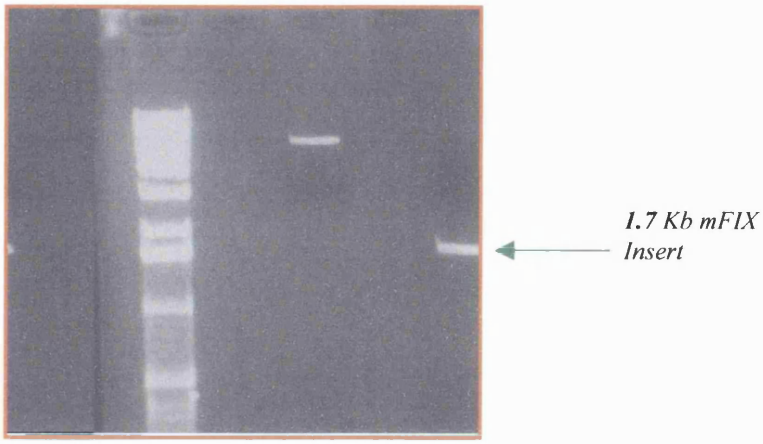
The resulting vector produced is shown in the plasmid map below:

Figure 13 FVII/PCDNA-3 vector



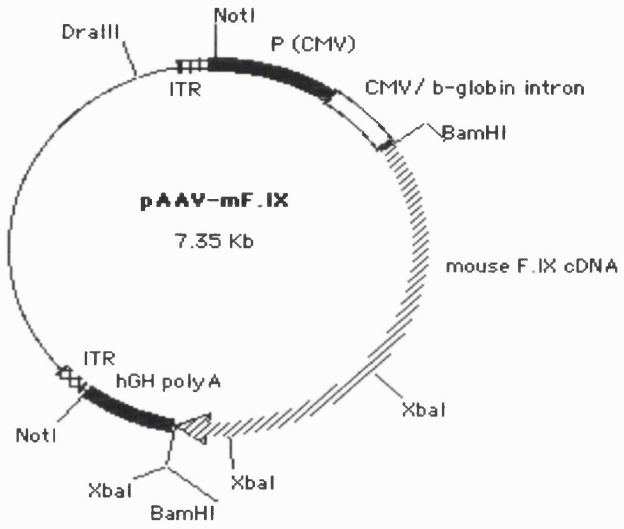
4.1.2 AAVmFIX construct

In order to perform experiments with a species-specific homologous transgene a murine FIX AAV plasmid was constructed. A 1.7Kb mFIXcDNA insert was removed from its supply plasmid as shown below:



The insert was then excised from the gel and the DNA extracted by a gene clean method. The insert was ligated and cloned into an AAV-2 plasmid which resulted in a plasmid containing mFIX under transcriptional control of a cytomegalovirus IE enhancer/promoter, a chimeric CMV/ β globin mini intron (5' to the mouse FIX cDNA), and the human growth hormone polyadenylation sequence. The plasmid map is shown below:

Figure 14 *AAVmFIX plasmid*

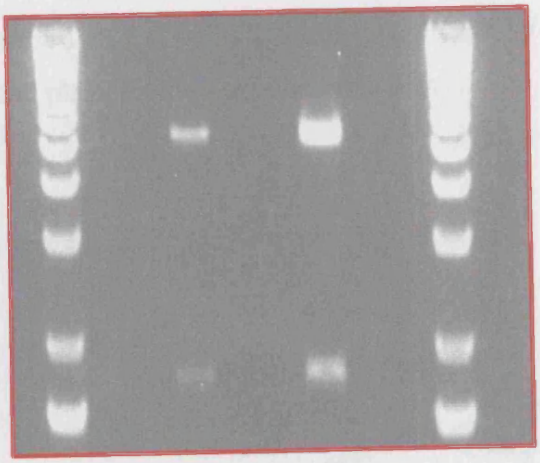


The AAV mFIX plasmid was then grown up in a maxiprep (Quiagen) (as described in materials and methods Chapter 3, page 79) and stored in glycerol buffer at -70°C.

4.1.3 AAVhFIX3 construct

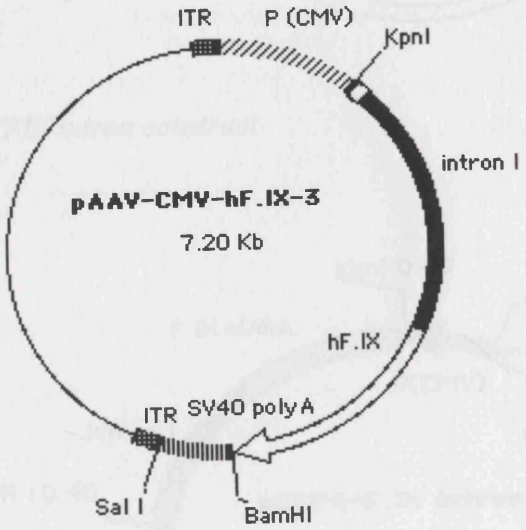
The AAV hFIX construct used for these experiments was gift to the laboratory from Avigen Inc (California, USA). This construct has a CMV promoter and contains the intron 1 of factor IX gene. The construct integrity was checked by performing a restriction digest to release the 1.9 Kb factor IX cDNA insert as shown below:

*AAVhFIX3 Restriction Digest with Ecor-V Yielding **1.8/5.4 Kb** Fragments*



The resultant expression AAVhFIX expression vector used for some of the experiments is shown below:

Figure 15 *AAVhFIX3*



Further FIX expression vectors studied

Other vectors were used PCEPIV/ FIX intron and PCEP IV. These were produced some years earlier and plasmid maps are shown below:

Figure 16 *PCEP IV/FIX construct*

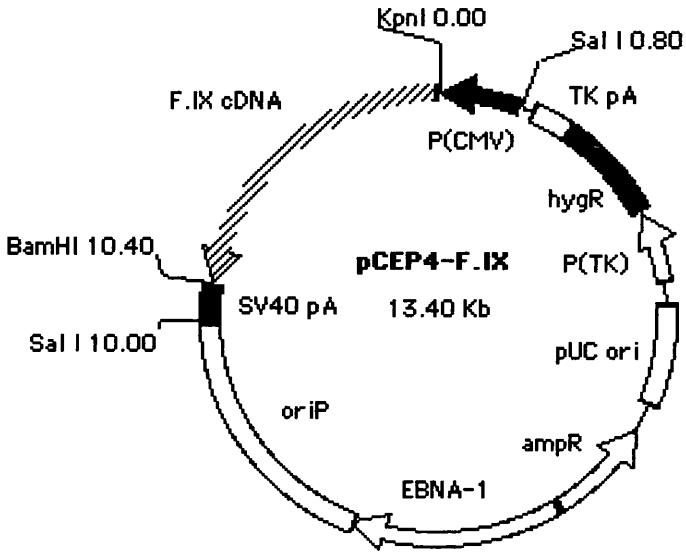
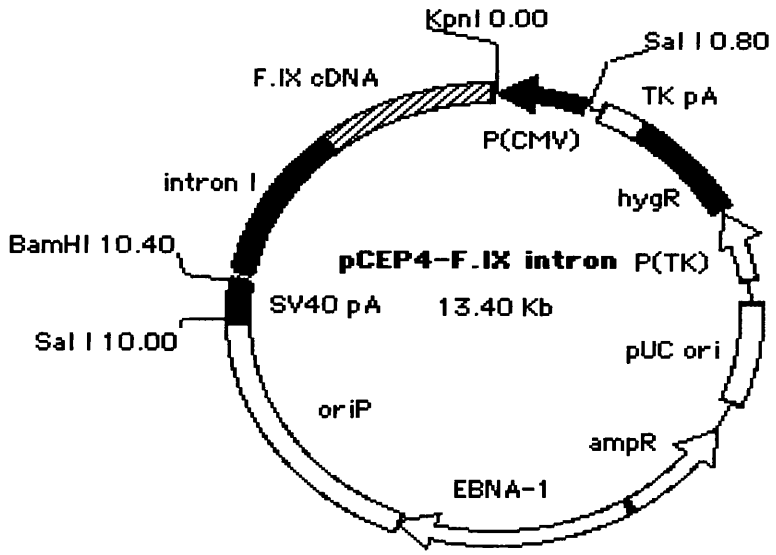


Figure 17 *PCEPIV/FIX intron construct*



4.2 *In Vitro Studies*

The vectors described above were tested *in vitro* in cell culture under the following conditions. Typically the *in vitro* tests were carried out using a standard transfection technique incorporating the use of lipofectamine (see materials and methods chapter 3, page 80).

Method

For the FVII plasmid transfections, the reactions were carried out in 6 well plates at 60-70% cell confluence. The ratio of (DNA vector : Lipofectamine) was 1:3 for all experiments. For the FIX studies with the three vectors were used a) pFIXCEPIV/intron b) pFIXCEPIV, c) pAAVFIX. The ratios of DNA vector to lipofectamine is shown in the table below: The FIX constructs were then tested out in C2C12 cell lines as shown below.

Vector	DNA Vector	Lipofectamine
PFIXCEPIV intron	**6μg**	**18μg**
PEmpty Vector	**6μg**	**18μg**
Mock	-	-
PFIXCEPIV	**6μg**	**18μg**
PEmpty Vector	**6μg**	**18μg**
Mock	-	-
PAAVFIX intron	**6μg**	**18μg**
PEmpty Vector	**6μg**	**18μg**
Mock	-	-

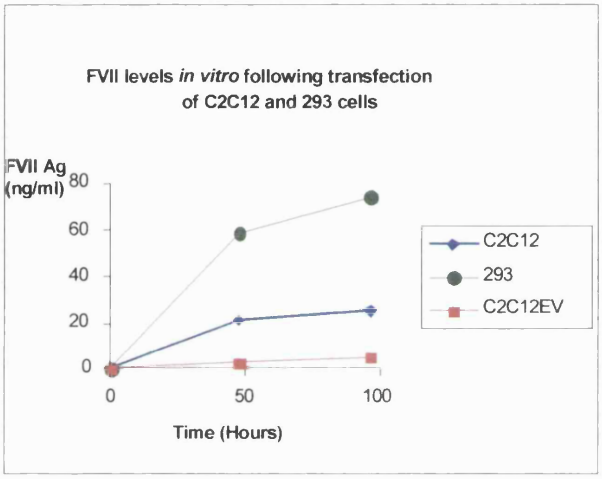
4.3 *In Vitro* Results

The FVII construct (PCDNA-3/FVII) produced was tested *in vitro* in C2C12 and Cos 7 cell lines. In order to study the transfection of target cells it is necessary to test the vectors with and without an insert, i.e. an empty vector.

4.3.1 FVII: C2C12

The results of the transfections are shown in the figure below. The graph shows the results for the vector containing the FVIIcDNA insert, and for the vectors with no insert (EV), which acts as the negative control for these experiments.

Figure 18 *FVII levels post transfection C2C12*

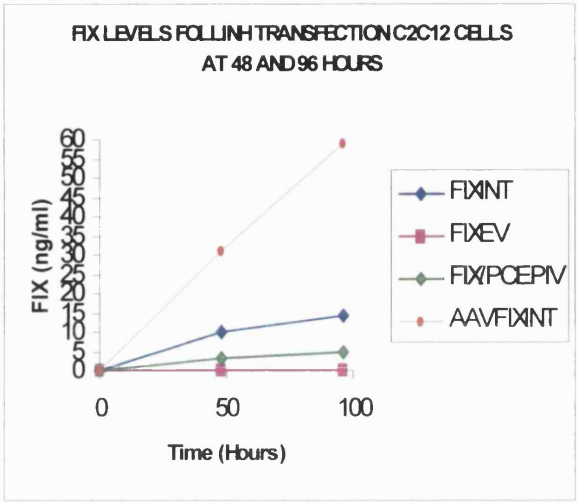


As can be seen for the FVII plasmid results, it was possible to transfect the two tested cell lines but the resulting FVII antigen levels were low. A better result was obtained in the 293-cell line.

4.3.2 FIX C2C12

The three vectors described above were tested in C2C12 cell lines and the results are shown in the graph below:

Fig 19 *FIX Ag post transfection C2C12*



Amount of FIX produced

In calculating the amount of factor IX produced per 24 hours the size of the plasmid has to be taken into account.

For **plasmid 1** (*pCEPIV/Intron*): 10µg/24/10^6 /cells

Started with 13.4 Kb **PCEPIV** plasmid using 6µg = 0.63pmol

Therefore produced 10µg per 24 hours from 0.63pmol

Amount = 14.7µgFIX/24HOURS / 10^6 cells/ pmol plasmid

For **plasmid 2** *(pCEPIV)*: 4µg /24/10^6 / cells

Amount = 5.9µg FIX/24HOURS / 10^6 cells/ pmol plasmid

For **plasmid 3** (*pAAVFIXintron*)

31µg/24/10^6 /cells

Started with 6µg 7.2 Kb plasmid =1.26pmol

Therefore 31µg per 24 hours from 1.26pmol

Amount = 24.6µgFIX/24hours/10^6 cells/pmol plasmid

Summary

As can be seen from the above, the best results were obtained with both the intron containing plasmids. The levels attained were considerably higher with the intron-containing construct, which was in keeping with previously published data (Kurachi *et al.*, 1995). It is presumed that intron-containing constructs stabilize mRNA available for translation of the FIX protein, hence improving production of protein. As increased efficacy was demonstrated with the intron containing constructs *in vitro,* these constructs were selected for *in vivo* use.

4.4 Viral Vector Production

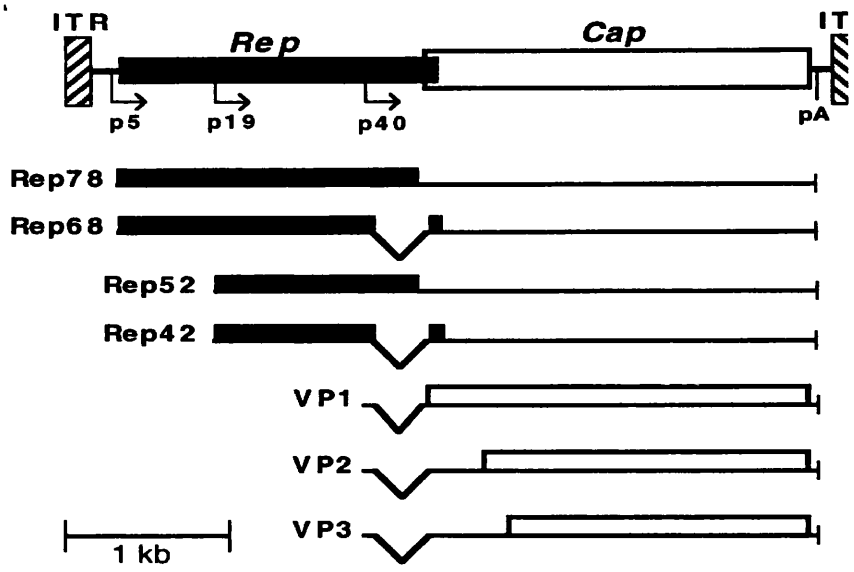
Adeno Associated Vector (AAV)

Adeno-associated viruses (AAV) are non-pathogenic, replication defective parvoviruses that usually require coinfection with a helper virus to generate new infectious virus particles. AAV infection in humans and other animals is asymptomatic and non-pathogenic. AAV has several advantages, when considering its potential use as a gene therapy vector. Infection is asymptomatic and AAV will infect a wide variety of cells. The 4.7 Kb genome of wild type AAV undergoes targeted integration on chromosome 19 after infection (Kotin *et al.*, 1990; Kotin *et al.*, 1991; Samulski *et al.*, 1991; Kotin *et al.*, 1992). Most recombinant AAV vectors do not include the *rep* gene (which would severely limit packaging capacity), and therefore, site specific integration is lost (Walsh *et al.*, 1992; Kearns *et al.*, 1996; Yang *et al.*, 1997; Russell & Hirata, 1998).

The pro-viral genome can be rescued for viral replication from pro-viral DNA and cloned into a plasmid, thereby making construction of recombinant genomes feasible. (Samulski *et al.*, 1982). AAVs are the smallest DNA animal virus, and the single stranded linear genome is encapsulated in an icosahedral structure, approximately 20nm in diameter. The organisation of the genome is as follows. There are two open reading frames (ORFs) .The ORF that extends over the 5' left half of the genome encodes for replication (*Rep*) proteins, and the 3' right encodes for structural (*Cap*) proteins. There are three promoters located at 5, 19, 40 map units respectively. The

genome is flanked by two inverted terminal repeats (ITRs) of 145 Bp (Koczot et al 1973, Lushby et al 1980).

Figure 20 *AAV genome*



Map of the wild-type AAV genome, including Rep (solid) and Cap (open) reading frames, promoters (p5, p19, and p40), polyadenylation site (pA), and inverted terminal repeats (ITR). The viral transcripts encoding the different Rep and Cap (VP1-3) proteins are shown below the genome. The smaller Rep proteins, VP2 and VP3, are translated from internal initiation sites.

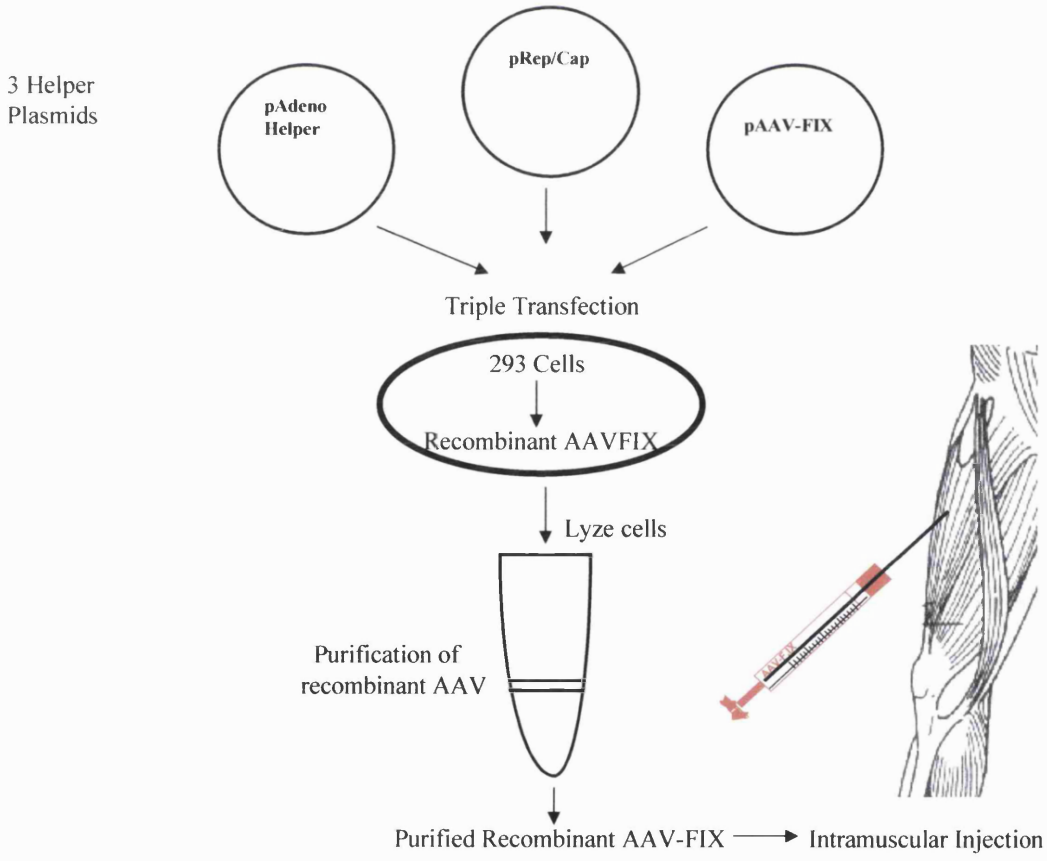
The terminal 125 bases are palindromic, but the overall palindrome is interrupted by two shorter palindromes in the centre of the larger palindrome, forming a T shape structure after maximum base pairing. This hairpin structure is used a primer for DNA replication. The ITRs do not appear to contain dominant promoter or enhancer activity. It has been shown that the ITRs are the only elements required in *cis* for integration, rescue, replication, and encapsulation of the AAV genome. Since the *Rep* and *Cap* proteins can be provided in *trans,* this has allowed the generation of viable rAAV vectors that can transduce a foreign gene into a target cells chromosome in a stable manner.

Principles of AAV Vector Production

AAV vectors are derived from the naturally occurring virus by recombinant DNA techniques. The AAV viral genome is cloned into a series of bacterial plasmids to

facilitate modification. The viral *rep* and *cap* genes are excised from the viral genome and cloned into AAV helper plasmid. The human factor IX gene is flanked by the inverted terminal repeats (ITRs) and cloned into an AAV vector plasmid. These plasmids are then used to transfect human embryonal kidney cells 293 in the presence of an adenoviral viral helper plasmid that contains the E2A and E4 genes and the VA RNAs from adenovirus type 2. Following the transfection with $CaCl_2$ co precipitation, the transfection buffer is replaced with serum free medium and the flasks are incubated further to get virus amplification. Following the incubation period the cells are removed from the transfection plates by gentle pipetting and are then pelleted by low speed centrifugation and resuspended in pre chilled 10 Mm Tris Cl pH 8. The cell extract can then be stored at – 80 ° C until the virus extraction. An outline of the procedure is shown below:

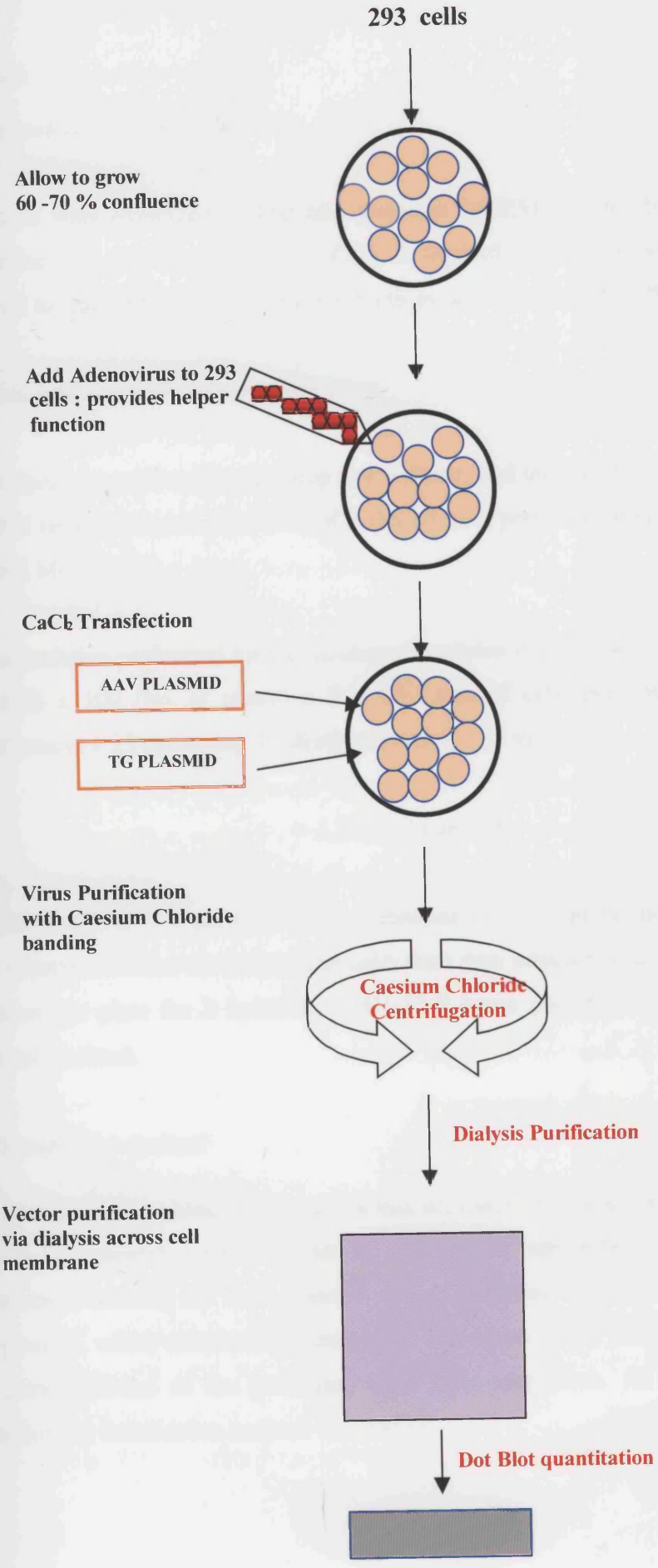
Figure 21 *Triple Transfection AAV*



4.4.1 Research Preparation made in the laboratory

In order to conduct the small animal experiments (murine) it was necessary to produce a recombinant adeno associated viral vector in the laboratory. Commercially, these vectors are produced by the triple transfection method as described above, and these methods are now used routinely in the laboratory as well. The first preparations made for the production of rAAV vectors were made with the use of an adenovirus providing the adeno-helper functions. In the later preparations, this was substituted with the use of an adenoviral plasmid. The method of this preparation is described below, which describes the protocol using an adenovirus to provide the helper functions. The process is laborious and takes approximately 1 month from start to completion. A diagram describing the process is shown below:

Figure 22 rAAV production with Ad helper virus



Method

a) Expansion of the HEK 293 cells

293 cells were expanded in 150 mm plates in DMEM/ 10 % FBS / 1% P/S 1%, glutamine (20 ml/plate), 37%, 5% CO_2. A total of 100 plates were prepared and allowed to reach 80-90% confluence. Infections were performed in 2% FBS / DMEM.

b) Viral Infection with Adeno helper virus

The helper virus utilised for the prep was Adlac z, and the infection was carried out at an MOI (multiplicity of infection) of 2 (based on a previous experiment to elucidate optimal MOI).

The calculation performed for the number of particles required was:
2 (MOI) x 100 (No of plates) x 5 x 10^7 (No of cells per 150mm plate) x 0.7 (confluency) x 25 (assuming 1 infectious particle / 25 virions)

$$= 1.75x10^{11} \text{ particles}$$

Therefore 8.75 x 10^{10} particles / litre of medium (1 litre will be enough for 50 plates) was required to infect the plates. The cells were then infected with 20mls of infectious medium per plate for 2 hours at 37°C. At 2 hours post infection the transfection cocktail is added.

c) Transfection cocktail

The transfection cocktail consisted of a mix of: $CaCl_2$, x2 Hepes buffer, and the DNA. The initial research preps consisted of preparing a transfection cocktail of the two plasmids containing the trans plasmid, which contained the AAV elements, and the *cis* plasmid, which contained the transgene. The adeno helper functions were provided by prior infection of the packaging cells with adenovirus. At 2 hours post viral infection the transfection cocktail was added.

Transfection cocktail mix

In 50ml conical tube:

4.5 ml 2M $CaCl_2$

750 µg *trans* plasmid (625µl)

250 µg *cis* plasmid (157µl)

Sterile $H_2 0$ to a final volume of 36 ml

In this case, the *trans* plasmid concentration was 1.2 µg/ml and the *cis* plasmid concentration was 1.6µg/ml. Therefore, for a 20-plate preparation, 625 µl of Trans and 157 µl of *cis* plasmid were used. So each conical contained 4.5ml 2 M $CaCl_2$ 30.7ml water plus the *cis* and *trans* plasmids.

In a 200mls conical tube, 36mls of x 2 Hepes was added, vortexed in the hood, adjusting the speed to avoid splashing. Then the $CaCl_2$ / DNA mixture was added drop wise from 25ml pipette while vortexing. The 72mls mixture was incubated for 25-30 minutes at room temperature. This mix of 72mls was sufficient for 20 plates. A total of 5 transfection cocktails were prepared at approximately 5 minute intervals, so that the first cocktail had been incubating for 20-25 minutes when the last cocktail was prepared. The cocktail was then shaken up after the incubation period was up and added drop wise onto the 293 cells using 3.5mls of transfection cocktail per plate. The plates were then returned to the incubator overnight at conditions of 5 % CO_2, 37°C. The following morning the transfection medium removed and replaced with DMEM 1% Pen /Strep.

The cells, which developed CPE after 48 hours, were harvested after 72 hours. To each plate, 0.5mls 0.5M EDTA, pH 8.0 was added. The cells were harvested with a 10 ml pipette and transferred to an autoclaved 500mls centrifuge tube. The tubes were placed on ice and spun at 1000g for 20-30 minutes. The supernatant was decanted into a flask, which had been sterilised with bleach. The cells were resuspended in cold sterile Tris 10 mM, pH 8.0. The final volume of the cell suspension was 30-40ml and was frozen at -80° C before isolation of the AAV vector.

d) Virus extraction from transfection plates

The cell extract was then lysed in order to release the virus. This was performed by 3 1-minute rounds of sonication at an output of 2.5. Once the sonication was completed, RNAase was added to a final concentration of 0.2mg/ml. Additionally Bovine Pancreatic DNAase I was added; for the 50 plate prep, 5mg of lyophilised powder was added. The sample was gently mixed and incubated at 37°C for 30 minutes. Sodium deoxycholate was then added to solubilise the membranes at a final concentration of 0.5%. The tube was immediately removed and placed in ice water for 10 minutes. The sample was then ready for virus purification by performing caesium chloride centrifugation.

e) *Caesium Chloride centrifugations*

Spin 1

For every ml of sample 0.454 grams of CsCl was added. The volume of the sample was measured with a 50-mls pipette. The volume is then made up to 40mls with 1.3 grams/ml CsCl. The sample was then ready for loading into the centrifuge tube. The bottom tier of the caesium chloride consists of 9ml 1.6 grams/ml CsCl. The middle tier consists of 9 ml 1.45 grams/ml CsCl. Finally, 20 mls of the sample is added to the top of the gradient. The tubes were spun at 25000 RPM at 4°C for 18-24 hours.

Harvesting the fractions

Using the Beckman recovery system, the first 12-ml of the sample was collected into a 12ml snap cap tube. Once the 12-ml has come through, fractions of 1ml are collected. Fractions are collected until the entire Adenoviral AD band had come through. The fractions are placed on ice and are subjected to a PCR analysis to confirm the presence of AAV vector. The AD band was found in fractions 14 and 15, and the AAV fractions 9-13.

Spin 2

CsCl at 1.41 grams/ml was added to a final volume of 12ml and spun in a 70.1 rotor, ultra clear heat seal tubes, at 60000RPM overnight. The first 4 fractions were collected in 1ml fractions, and then as 0.5ml fractions.

Analysis of the fractions

Once the relevant fractions were collected, the remaining fractions were discarded. The refractive index for the remaining fractions was calculated using a refractometer using a total of 6 µl of sample to analyse the refractive index. The density of the fraction was calculated using the formula:

$$\boxed{\textit{Density (gm/ml)} = \textit{RI x 10.8601} - \textit{13.4974}}$$

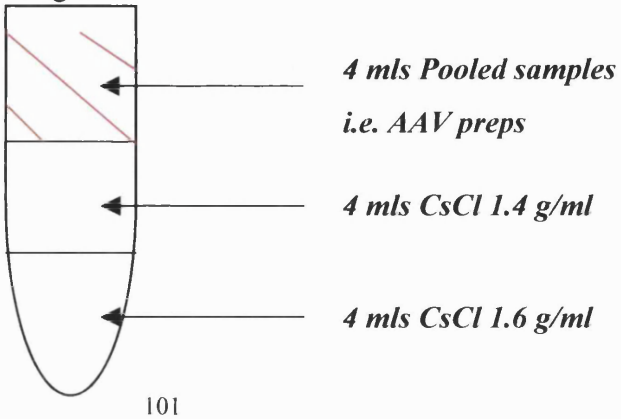
Centrifugation (1994) : D Rickwood, T C Ford, J Speensgeard (John Wiley)

The desired fractions containing the AAV particles should fall within a density range of 1.41 to 1.38 gm/ml. At this stage 1 µl of each fraction with a density of 1.35- 1.43 gm/ml is removed and mixed with 1 ml H_20 and was used for later PCR analysis (1µl)

Spin 3

The fractions from the second centrifugation with densities of 1.38-1.41 (i.e. where AAV fractions are) were pooled. This was done by pooling 0.5-ml samples (total 2ml) and adding 2 mls of 10 mM Tris pH 8.0 to make a final volume of 3.8-4ml. A gradient was set up as follows in an ultra clear 12-ml centrifuge tube (SW41). 3.9 mls of 1.45g/ml CsCl (in 10 mM Tris, pH 8.0, filter sterilised) was added to each tube.

Slowly the sample was underlayed with 3.9 ml 1.6gm/ml CsCl (in 10 Mm Tris, pH 8.0, filter sterilised). The ultra centrifuge was cooled prior to loading up the samples. The pooled fractions were then loaded onto the gradient. The centrifuge tubes were placed into cooled SW41 rotor buckets. The ultracentrifuge was balanced and started up to spin at 35000 RPM at 4°C overnight. The tube below represents the layered gradients prior to centrifugation. The tube is then loaded into the Beckmann rotor and the rotor balanced prior to centrifugation.



At the end of the third centrifugation, the fractions were collected initially in 1 ml fractions and then in 0.5 ml fractions. Subsequently for each fraction the refractive index was measured and the density for each fraction calculated. The AAV fraction (vector) will be contained in the fractions with the density ranging between 1.36g/ml to 1.41g/ml. (The fractions collected with densities corresponding to these readings were fractions 9,10, and 11, *see table 4, p105*)

f) Dialysis step

Subsequently the selected AAV fractions were subjected to dialysis, the principle being to remove any residual caesium chloride contaminating the fractions. This was done by dialysis extraction of vector by taking the pooled fractions (i.e. 9,10,11) containing the bulk of the AAV vector and injecting into a slide a lyzer cassette dialysis cassette (Pierce, 0.5-3ml, MWCO = 10,000). Prior to injection some of the air from the cassette was removed with an 18 gauge needle / 3 ml sterile syringe. The pooled fractions were then injected into the cassette using a different hole. A flotation device was attached to the cassette, and the cassette placed in a cold Hepes buffered Saline (HBS) in a beaker, pH 7.8 in cold room (600- 800ml (HBS) in beaker). The mixture was stirred at slow speed (using a magnet) at 4°C for 6 hours, changing the buffer every 2 hours.

Finally the vector suspension was removed from the cassette with a fresh syringe using an 18-gauge needle, and transferred to a sterile eppendorf tube in the hood and the volume determined, and the tube placed on ice. The vector was stored with a 5% volume of heat sterilised glycerol (microwaved for 20-30 sec) ensuring the vector is mixed well. The vector was then distributed to cyrovials (200 µl / cryovial) and eppendorf tubes. The vector was freezed in dry ice and placed in – 80 ° C freezer until required for future analysis.

Volumes obtained post dialysis

	Volume	Glycerol vol (5%)
Fraction **9**	560µl	30µl
10	520µl	28µl
11	460µl	25µl

g) Dot –Blot quantitation to caclculate rAAV concentration

From each fraction (i.e. 9,10,11) 5 µl was kept to perform a dot blot quantitation. The 5µl samples were then for prepared for the dot blot quantitation procedure to estimate the amount of vector generated.

Labelling of radioactive probe

Prior to the dot blot quantitation, a probe was labelled according to the protocol using a random primer labelling kit (Strategene, Primeit II, US). 10µl of DNA (10ng/ul) was taken and mixed with a primer 10µl and water 14µl. The mixture was heated for 5 minutes in boiling water and centrifuged; this opens up the DNA for labelling. Then 10µl of x5 buffer, 5µl of p32 labelled (dCTP), 1µl Klenow (DNA polymerase) was added to the mixture making a total of 50µl .The mixture was incubated at 37-40 ° C for 2 to 10 minutes. At the end of the incubation, the mixture was checked for radioactive activity to see the probe had been labelled. The probe was subsequently placed in the radioactive fridge.

Extraction of DNA from AAV fractions

2µl of sample was diluted into a 178 µl of water. Then 20 µl of x 2 proteinase K was added and the mixture and incubated for 1 hour at 37° C. The mixture was then allowed to cool to room temperature, and subjected to a Phenol/Chloroform- isoamyl alcohol (25:24:1). Extraction was carried out with chloroform-isoamyl alcohol (24:1). 8µl of glycogen (5mg/ml, Ambion), 20µl 3 M sodium acetate, pH 5.2, and 450µl ethanol. Precipitation was then carried out at - 80° C for 1 hour. The mixture was subsequently spun at maximum speed in the cold room for 20 minutes and the supernatatant was carefully removed. The pellet was dried in a speed vacuum and resuspended in x1 T.E buffer (10µl). Then 5µl, 2.5µl, 1µl, and 0.5µl was added to 150µl of spotting solution respectively. Serial dilutions of plasmid standards (5µl, *c*3.2ng/ml) were made with the spotting solution (150µl). Each plasmid standard covered the equivalent of 20ng, 10ng, 5 ng, 1ng and 0.5ng DNA each resupended in 5µl.

Slot blot hybridisation

Once the standards (x6) and test samples (x6) were made up in the spotting solution (0.4M NaOH, 25mM EDTA, 0.00008% Bromothymol blue), the next stage was to prepare the membrane, which was used in the binding of the DNA. The spotting solution is alkali and denatures the DNA (-vely charged) to allow it to bind to the positively charged membrane. The pre cut Biodyne membrane (0.4M NaOH, 25mM EDTA, 0.00008% Bromothymol blue), is wetted along with pre cut filter paper for 1-30 minutes. The slot blot apparatus is assembled and a vacuum applied. The standards and samples are then applied and the vacuum turned off once the samples have passed through. The membrane is subsequently transferred to a hybridisation tray (Perkin Elmer) containing 100ml pre warmed pre hybridisation solution. The mixture is placed in a shaking water bath at 50°C for 20 minutes. At the end of this period the pre hybridisation buffer was removed and 30 mls of hybridisation solution and the denatured probe were added. The mixture was incubated in a shaking water bath for 20 minutes. Subsequently the membrane was rinsed in 100 mls pre warmed wash solution. A wash was then carried out in 100mls wash solution at 65°C for 10-15 minutes and two further rinses were carried out for 1-2 minutes in 100 ml of wash solution. The membrane was then placed in a seal a meal bag removing the excess liquid and placed in an X-ray cassette. The film was exposed at – 80° C overnight and the film developed the next day.

PCR Reactions

The PCR reactions were set up as follows: 2µl per dilute fraction as template, primers specific for vector. For the PCR beads: 2µl template. 0.5µl forward primer (50 Um). 0.5µl reverse primer (50 Um), 22µl H_20. PCR reaction: 94 ° C for 5 minutes, 94 ° C (1minute) / 52-53 ° C (1 minute) for 25 cycles, 72° C for 7-10 minutes. The PCR products were run out on a 1.5- % agarose gel and visualised. The lanes were labelled with their corresponding fractions.

4.4.2 Results

Density gradients

The table shown below gives the density results obtained from the second and third centrifugation spins. The refractive index for each fraction is measured using a refractometer and the density calculated according to the following formula:

Density = RI x 10.8601- 13.4974

Centrifugation (1994) : D Rickwood, T C Ford, J Speensgeard (John Wiley)

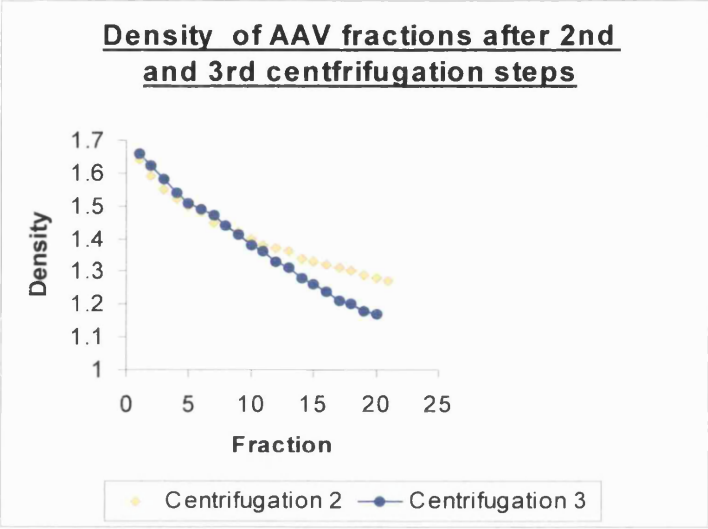
Table 4 *Densities centrifugations 2 & 3*

Fraction	RI	C2	RI	C3
1	1.3936	1.64	1.3957	1.66
2	1.389	1.59	1.3917	1.62
3	1.3857	1.55	1.3881	1.58
4	1.383	1.52	1.3846	1.54
5	1.381	1.5	1.3821	1.51
6	1.3791	1.48	1.38	1.49
7	1.3768	1.45	1.378	1.47
8	1.375	1.44	1.3755	1.44
9	1.3737	1.42	1.3731	1.41
10	1.3716	1.4	1.37	1.38
11	1.3699	1.38	1.3677	1.36
12	1.369	1.37	1.3654	1.33
13	1.3679	1.36	1.3635	1.31
14	1.3664	1.34	1.3606	1.28
15	1.3657	1.33	1.3592	1.26
16	1.3644	1.32	1.3566	1.24
17	1.3635	1.31	1.3547	1.21
18	1.3626	1.3	1.3531	1.2
19	1.3617	1.29	1.3519	1.18
20	1.3611	1.28	1.3755	1.17

Density results of the AAV fractions from 2nd and 3rd centrifugation steps

The following plotted graph shows the results obtained for the densities of the second and third centrifugation steps:

Fig 23 *Densities post 3rd centrifugation*



PCR 2ND Gradient AAV Prep

The gel represents the PCR analysis for examination of the fractions 9 to 13. The numbers represent the fraction numbers.

Fig 24 *PCR AAV fractions*

DNA Ladder 9 10 11 12 13

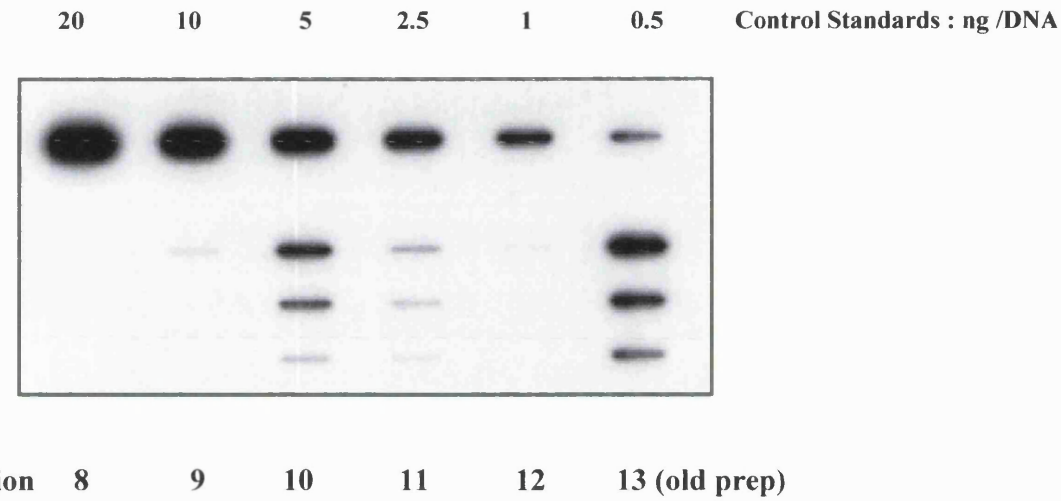
From the predicted density results fractions 9 to 12 were analysed by a PCR reaction to confirm the presence of AAV vector in the fractions. The above gel shows a very strong signal obtained for fractions 11 and 12. These bands correlate strongly with the

predicted bands from the density results. Although not clearly shown here, bands 10 and 13 were also weakly positive. Therefore preps 10,11,12,13 were harvested and loaded for the third centrifugation.

Dot blot quantitation

From the dot blot quantitation the amount of vector produced from the prep is calculated.

Fig 25 *Dot Blot*

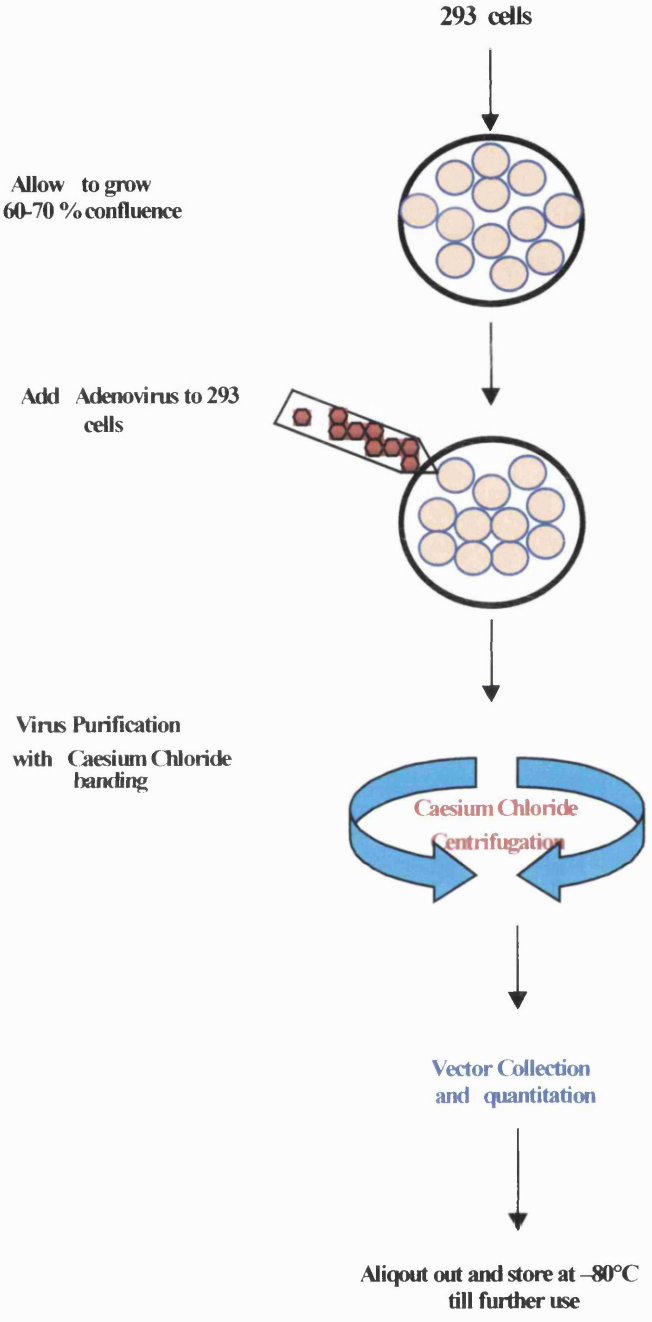


The dot blot above shows that fraction 10 at 1 µl volume is approximately half the intensity of the control standard at 2.5 ng (1ul volume) .1ug DNA approximates to 5Kb equating to 0.3 pmol. Therefore 2.5 ng of 7.3 Kb plasmid equates to 5.1 x 10^{-4} pmol = 5.1 x10^{-16} mols. Therefore 6.023 x10 ^{23} x 5.1 x10^{-16} = 3x 10^{8} plasmid particles = 6 x10^{8} vector genomes per µl = 6x10^{11} /ml

4.5 Adenovirus Preparation

The stages required in an Adenovirus preparation are relatively straightforward when compared to the AAV preparation. The stages required are infection of 293 cells with the adeno virus in question, and then incubation prior to virus harvest and purification through caesium chloride banding. The stages are outlined in the following diagram:

Figure 26 *Adenovirus production*



4.5.1 Method

a) *Preparation of HEK 293 cells*

To make an adenoviral vector HEK 293 cells (provided by the vector core at the University of Pennsylvania) were grown out and split into 150 mm petri dishes using 25 ml DMEM, 10 % FBS, 1% P/S for each plate. After reaching confluence the cells were further split and expanded.

b) *Infection of cells*

Each 150 mm dish requires a total of 1x 10^{10} particles of virus suspended in 10 ml DMEM, 1% Pen/ Strep (serum free). A total of 5 x 10^{11} particles were suspended in 1/2 litre of medium. (enough to infect 50 plates). The medium was aspirated from the dishes and 10 mls of medium containing the virus was added to the cells in each plate. The incubation was allowed to proceed for 2 hours at 37 ° C. At 2 hours 15mls of DMEM + 10% FBS + 1% Pen/ Strep was added to each plate. After 30 hours the cytopathic effect (CPE) was visible (the cells appear round and refractile and begin themselves to lift off the plate with very little agitation). At this stage by pipetting the media over the growth surface the cells were removed from the plate. The cells were collected in into 1-litre autoclaved centrifuge bottles. The bottles were centrifuged at 4000RPM for 10 minutes at 4° C. The supernatant was removed into bleach being careful not to dislodge the pellet.

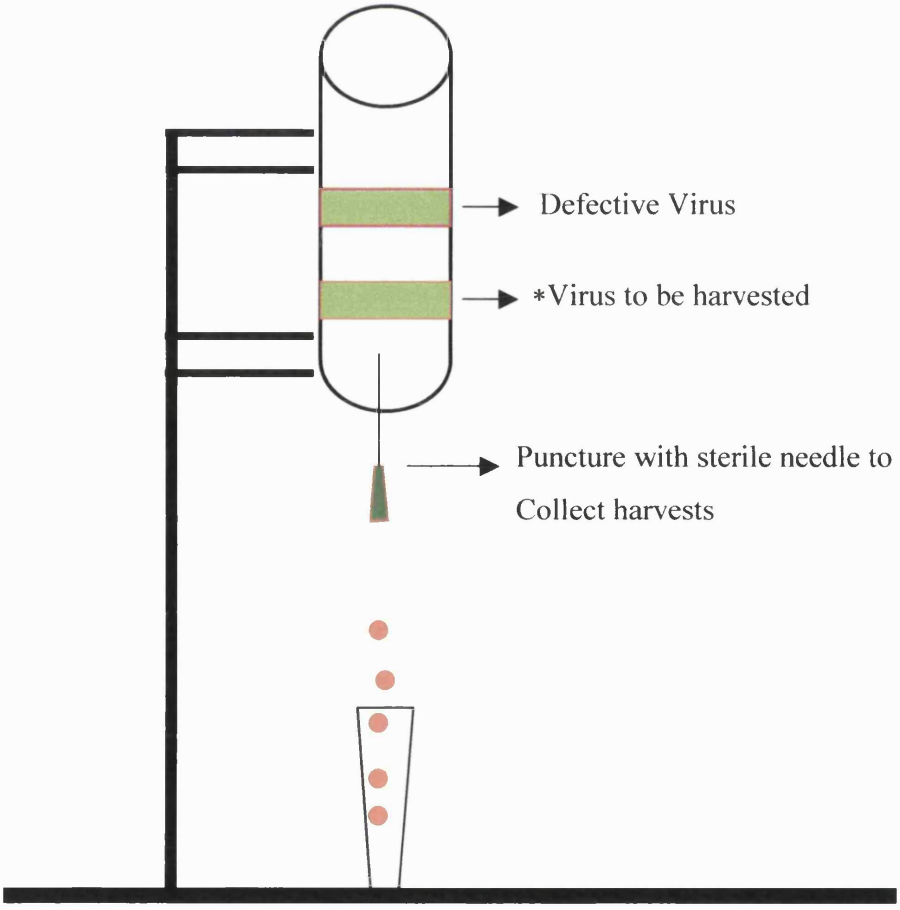
Virus Extraction

The pellet was resuspended in 10mM Tris HCL (pH 8.1), using 0.5ml of Tris per plate harvested. (At this stage the pellet may be stored prior to the next stage of the procedure). The pellet was then freeze thawed three times in alcohol dry ice / 37 ° C to release the virus from the cells. The lysate was subsequently spun for 10 minutes at 3000 rpm at 4° C. The supernatant is saved avoiding the pellet. The pellet was then resuspended in a small amount of 10-mM Tris –HCl, pH 8.1 to rinse the cell debris of any residual virus. The solution was then respun for 10 minutes at 4° C and the supernatants are then pooled together.

c) *Caesium chloride layering*

10 ml of light caesium chloride solution was pipetted into an ultracentrifuge tube of a Beckmann SW 28 rotor. Slowly and gently the light Caesium chloride (1.45g/ml) was underlayed with a solution of heavy caesium chloride (1.6g/ml). The light caesium floats to the top of the heavy caesium and the interface between the two layers should be very sharp. The viral supernatant was then carefully layered onto the top of the caesium gradient and the tubes were loaded into a SW 28 rotor ensuring the tubes were carefully balanced. The tubes were then spun for 2 hours at 20000 rpm at 4° C. The centrifuge tube was removed from the centrifuge and clamped onto a ring stand in a sterile fume cupboard. The virus appears as a narrow opaque white band 2/3 of the way down the heavy- light caesium chloride gradient. The tube is then punctured under the band with a sterile needle to allow the caesium chloride to drip out until the band is collected. Care is taken not to collect any of the bands above.

Figure 27 *Collection procedure for Virus*



d) Viral Extraction

The collected bands (2mls) is then diluted with an equal volume of 10 mM Tris (2 mls). In an ultra centrifuge tube (a Beckmann SW 40) 4ml of light caesium chloride is added. A further 4ml of heavy caesium chloride is carefully layered under the light caesium chloride. Finally the 4 mls of viral supernatant diluted in Tris is added on top of the gradient. The tubes are then loaded into the SW40 rotor and were spun overnight at 20000 rpm at 4°C. The Adenoviral band is then collected by puncturing the side of the tube with a needle and syringe. The band is then desalted over a Hepes column manufactured by Biorad (Econ Pac 10DG column, 7322010) and fractions of the collected bands are diluted 1:50 and an OD reading 260nm is recorded.
The amount of particles per ml is then calculated by the formula:

$$OD\ 260 \times Dilution \times 10^{12} = particles/ml$$

Yield of Fractions collected (OD (260 nm)

Fraction 1	0.053
Fraction 2	0.0157
Fraction 3	0.017
Fraction 4	0.0.17

Fraction 1 = $0.053 \times 50 = 2.63 \times 10^{12}$ /ml
Had 0.5ml (added 25µl glycerol), therefore aliquots made of 40µl = 1×10^{11} particles / eppendorf

Fractions 2,3,4 (were pooled together)
Had 1.52 ml (add 80µl glycerol), Aliquots of 125µl = 1×10^{11} particles / eppendorf
Glycerol was added to a final concentration of 5% and at stored at -80°C once aliquoted out.

Summary

The work described above shows that the production of recombinant viral vectors is not a trivial procedure. The process involves several intricate methods, and often the final outcome of a procedure that may last 4-6 weeks is not known right until the point of vector purification and yield quantitation. The early stages of rAAV production required the use of an Adenovirus as a helper virus. The procedure in the laboratory has now been modified because of fears of viral contamination with Adenovirus, and this incoporates the use of an Adeno viral plasmid providing the helper functions instead of an Adenovirus (Matsushita *et al.*, 1998). Other issues in recombinant vector production include obtaining a satisfactory yield of vector. Newer ways of vector purification have been developed to improve this process, such as using High performance liquid chromatography to purify the vector.

CHAPTER 5

GENE BASED TRANSFER TO MICE: PARAMETERS AFFECTING THE IMMUNE RESPONSE

5.1 Introduction

In order to demonstrate a proof of principle for a gene therapy concept it is necessary to demonstrate efficacy in a small animal setting before progressing to a large animal model. At the time of commencing these studies no animal models of the disease were available. Consequently, initial studies were performed in normal mice, both immune competent and incompetent. As outlined earlier, the immune response observed to a gene-based approach may involve separate responses to each component of the system i.e. to either the vector or transgene or both (Chapter 2, page 58).

Experimental design must therefore address each component in turn to dissect out the possible immune responses, which may be observed. The initial experiments in this chapter examine the response to vector selection. The three vectors used were Plasmids, Adenovirus (Ad) and adeno-associated vectors (AAV). The early studies were performed in normal immune competent mice. With the development of murine models of Haemophilia B, these studies were repeated in these affected mice. When these studies were conceived, there were no assay systems in place to study cellular responses (i.e. CTL response) to vector selection. Consequently, the work in this chapter makes use of antibody isotype profiles generated to different vectors to investigate the type of immune response that occurred to each vector. These results are informative since previous murine studies had defined that different isotype profiles correlate with different immune responses and in particular whether the CD4 T helper response was a Th1 or Th2 driven response (Severinson *et al.*, 1982).

The second set of experiments was designed to study the effects of transgene selection on the immune response. Prevoiusly published work addressing the immune response to the transgene product, involved the use of non homologous reporter genes such as Beta Galctosidase (a bacterial gene) encoding a *non secretable* transgene product (Yang & Wilson, 1995; Yang *et al.*, 1995c,(Fisher *et al.*, 1997) . Therefore, it would be conceptually difficult to extrapolate these findings to Haemophilia B, where the desired transgene product is *secretable*. Within the context of this part of the study three different transgenes (human, murine, and canine) were used in both murine and canine models of Haemophilia B.

The objectives of this work were to:

a) To characterise the humoral response to transgene product with emphasis on the temporal appearance of antibody formation

b) The nature and specificity of antibodies produced

c) To study the effect of underlying mutation on the resulting immune response

5.2 Vector Selection

Experimental design

Three cohorts of mice were injected intramuscularly with one of three vectors encoding human FIX as described below. At 2 weekly intervals, mice were bled and the serum harvested for analysis of FIX Ag expression and antibody production to human FIX Ag by subclass specific Elisa. At 12 weeks the mice were sacrificed and the injected limbs analysed histologically for FIX Ag expression, and for evidence of inflammatory infiltrates determined by Haematoxylin and Eosin staining, and immunoflouresence for CD8 infiltration. In further injected cohorts some mice were sacrificed at earlier time points to study the injected limbs histologically. For comparison with a non-secretable transgene a further cohort of mice were injected with Adβ gal and AAV β gal.

Animals

Normal 8-week-old C57BL/6 male mice (Charles River Breeding Laboratories) were anaesthetised by inhalation of mefofane, and a 1cm incision of the skin of one hindlimb was made. Three cohorts of C57BL/6 mice received IM injections of AAV (n=10), E1/E3-deleted adenoviral (n=7), or plasmid (n=8) vector at a single time point (numbers of animals reflect the sum of two independent experiments). The viral and plasmid vectors (diluted in sterile PBS) were injected with a Hamilton syringe at two IM sites (25 μl into tibialis anterior and 50 μl into quadriceps muscle). For AAV and

adenoviral vector, $4x10^{10}$ viral particles were injected per animal. 100 μg DNA was infused for the plasmid vector. The skin was closed with a suture. The mice were bled retro-orbitally on a biweekly schedule up to 12 weeks post-injection using heamatocapillary tubes.

Vector Preparations

All vectors contained an expression cassette containing the human FIX cDNA and the CMV IE enhancer/promoter. Similarly, the vectors encoding the β gal transgene were contained in an expression cassette containing CMV IE enhancer/promoter. The vectors used for these experiments were produced by the methodology described previously (Chapter 4, page 96-103). The plasmid vectors were made in large quantities by using the maxi prep (Quiagen) method as described. (Chapter 3, page79).

Assays for human FIX antigen and anti-hFIX

An ELISA specific for hF IX in murine plasma samples was carried out as described (Chapter 3, page 66)(Walter *et al.*, 1996). Antibodies against human FIX were detected by subclass specific ELISA (Chapter 3, page67).

Histological Analysis

Mouse muscle tissue was snap-frozen in liquid nitrogen-cooled isopentene and stored at -80° C till the sections were ready to be analysed. Cryosections (5-6 μm) were prepared and stained with haematoxylin/eosin (H&E) or with a goat anti-human FIX for immunofluorescent detection. Immunohistochemical detection of $CD8^{+}$ cells was carried out as detailed (Chapter 3, page 75)

Results

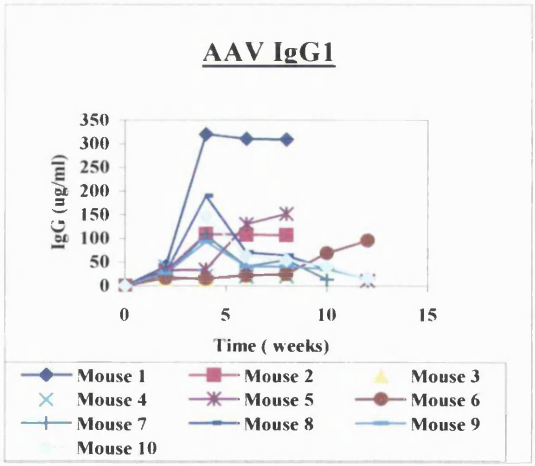
Antibody profiles

All of the injected mice developed anti-hFIX by 2 weeks post-injection which was maximal by 4-8 weeks. Analysis of the antibody isotypes revealed that all three

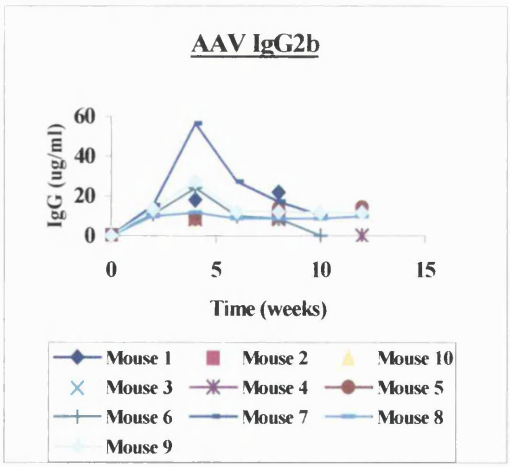
vectors induced antibodies of the T helper cell-dependent subclasses IgG1, IgG2b, and IgG2c). T helper cell-independent antibodies (IgM and IgG3) were not detected. C57BL/6 mice produce IgG2c instead of the highly homologous IgG2a subclass (Jouvin-Marche *et al.*, 1989a; Jouvin-Marche *et al.*, 1989b; Morgado *et al.*, 1989). Human FIX antigen was undetectable in plasma samples by ELISA with the exception of two adenovirus-injected mice that showed transient antigen levels during the first month after vector administration (*data not shown*). The results for the AAV injected animals are demonstrated in *figure 28*. For these animals, the most dominant isotype profile induced was an IgG1 profile (*Ratio IgG1/IgG2b =5.1*). The AAV-treated mice showed the lowest proportion of class IgG2 production of the three injected cohorts. Furthermore, 3 of 10 AAV-injected mice had undetectable IgG2c anti-hFIX while all adenovirus or plasmid injected mice developed IgG2c. The results suggest a bias towards an IgG1 profile in the AAV injected cohort.

Fig 28 *i) Antibody Isotype profiles of AAV injected animals*

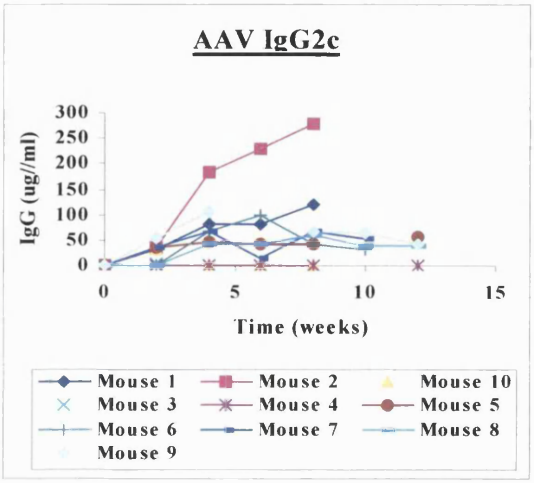
A)



B)

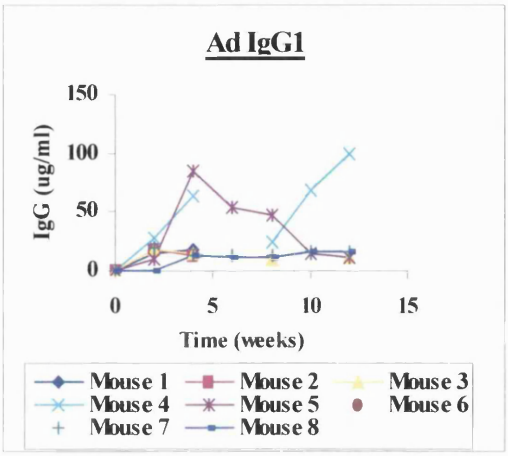


C)

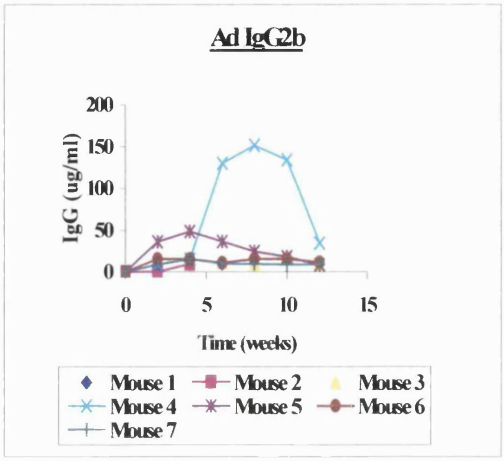


ii) Antibody profiles of Adenoviral injected Animals

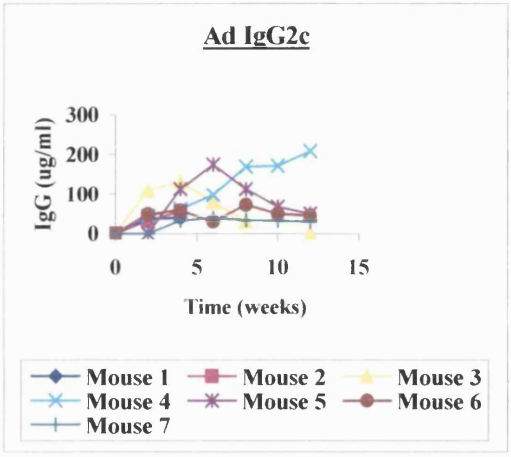
A)



B)



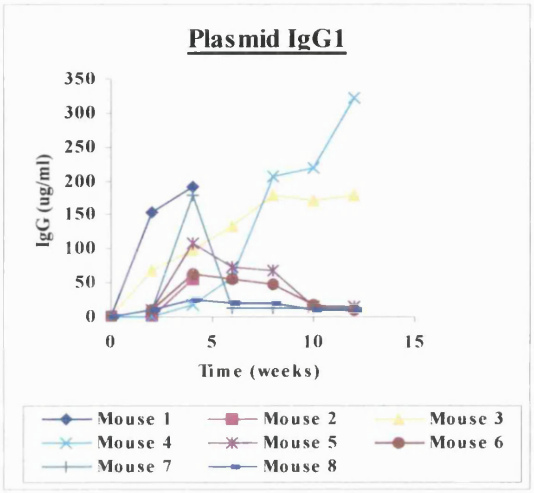
C)



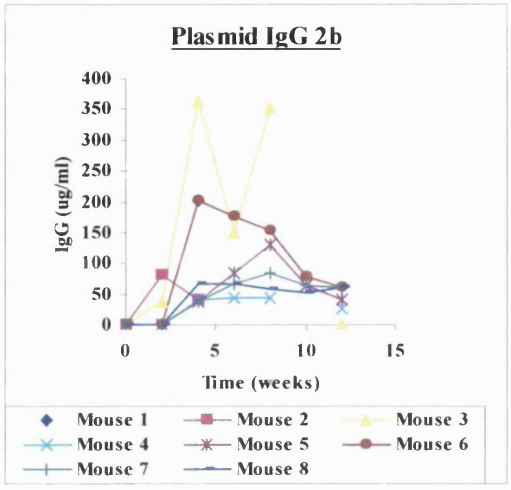
The results for the Adenoviral injected animals are shown in figure 28 ii). The Adenovirus injected animals displayed the most dominant IgG2 (Ratio *IgG1/IgG2b =1.1*) response with the lowest amounts of IgG1 subclass induction being shown for this cohort of animals.

iii) Antibody profiles of Plasmid injected animals

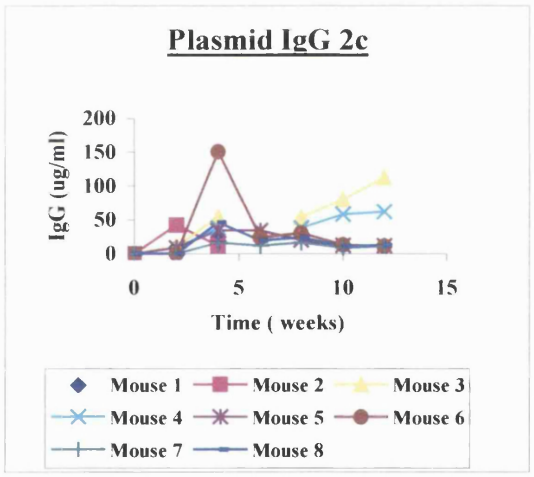
A)



B)



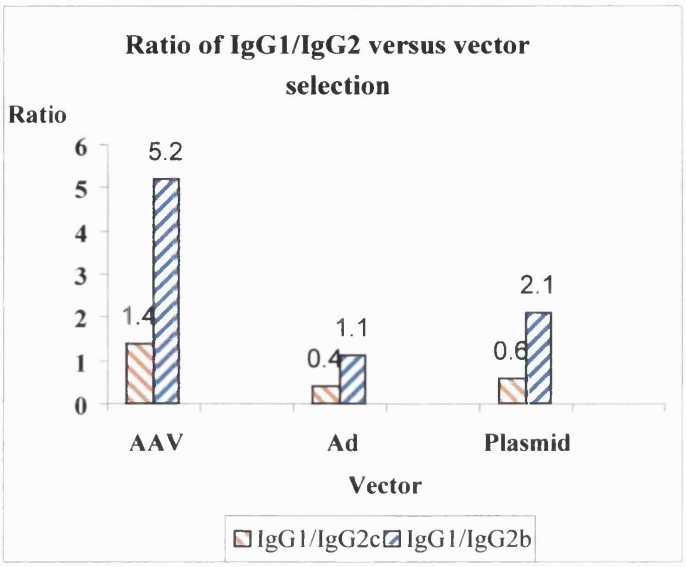
C)



The plasmid injected animals displayed high amounts of IgG1, which was intermediate between the Ad and AAV injected animals (*Ratio IgG1/IgG2b =2.1*).

The calculated ratios of IgG1 to IgG2b and IgG2c subclasses for all three injected cohorts are shown in the graph below at 8 weeks post injection.

Calculated proportions of IgG1: IgG2 subtype induction in three injected cohorts



The calculated ratio of the average antibody titre of IgG1 over IgG2b or IgG2c (at 8 weeks post-injection) shows a bias for IgG1 for the AAV vector-injected mice and for IgG2c for adenovirus-injected mice. Plasmid-injected mice showed an IgG1/IgG2b ratio subclass profile intermediate between AAV and adenovirus-treated mice, but displayed the overall strongest anti-hFIX immune response.

The data suggest that subsets of T helper cells activated by the secreted transgene product human FIX, might differ depending on vector selection. The data do not reveal any marked quantitative differences in the immune responses against hFIX because of variability in induction of IgG subclasses among different animals.

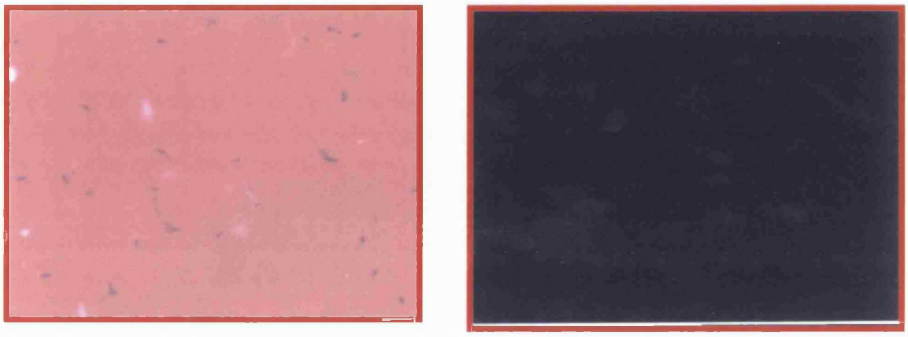
Histology of Injected muscle

1) Normal Muscle

Sections of muscle were removed from an uninjected animal and analysed for the presence of factor IX expression, inflammatory infiltrates by H& E staining (Chapter 3, page 73) and if indicated further staining by immunofluoresence for CD8 cell deposition (Chapter 3, page 75).

Fig 29 *Normal Muscle*

Haemotoxylin and Eosin *FIX Ag expression*



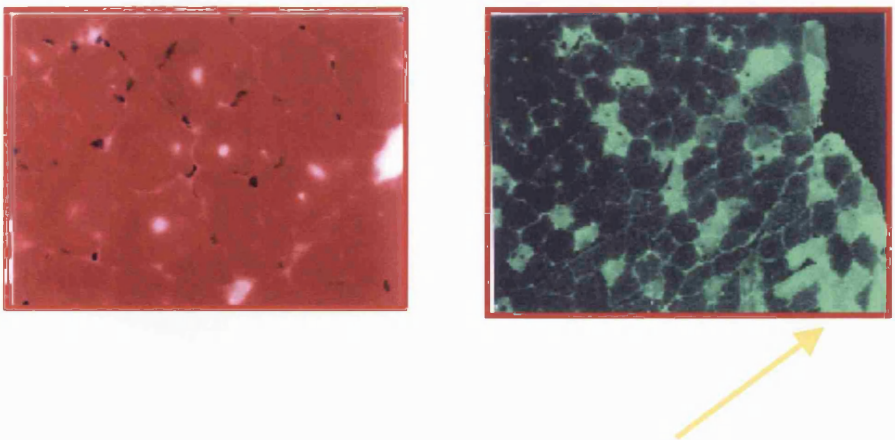
As can be observed from the appearances the muscle displays normal architecture with no disruption. The dark blue basophilic areas represent the muscle nuclei. No expression of human factor IX Ag is seen.

2) AAV injected animals

The histological sections for the AAV injected animals are shown below:

Fig 30 *AAV Injected animals*

Haemotoxylin and Eosin *FIX Ag expression*



FIX Ag expression

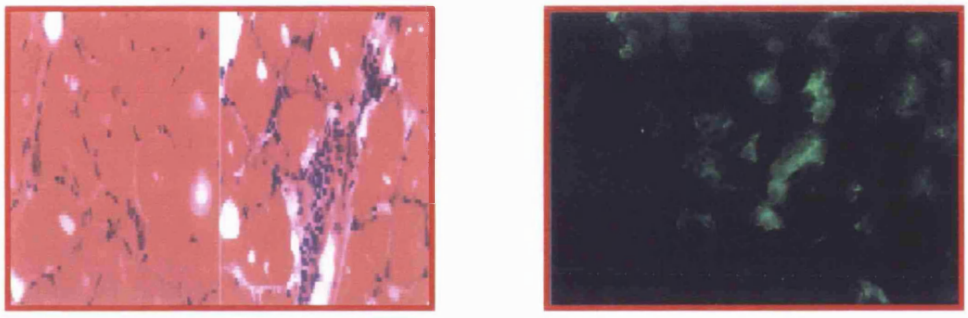
For the AAV based transduction clear expression of factor IX was seen as evident the immunoflouresent staining which is indicated by the yellow arrow (green fluorochrome (FITC) anti-factor IX antibody). No evidence of any inflammatory

infiltrates was seen, both on H & E staining and staining by immunofluorescence for the presence of CD8 and CD4 T cells.

Although the presence of cellular factor IX expression is evident histologically, no circulating detectable factor IX Ag is evident due to the formation of antibodies to the protein. The subclass of the antibody was predominantly of an IgG1 subclass (see earlier) indicating a predominantly a Th2 cellular driven response. It therefore appeared that long-term expression was achievable using an AAV vector with no cellular immune (CTL) responses being observed hisologically.

Figure 31 *Plasmid Injected animals*

Haemotoxylin and Eosin *CD8 expression*



Inflammatory Infiltrates

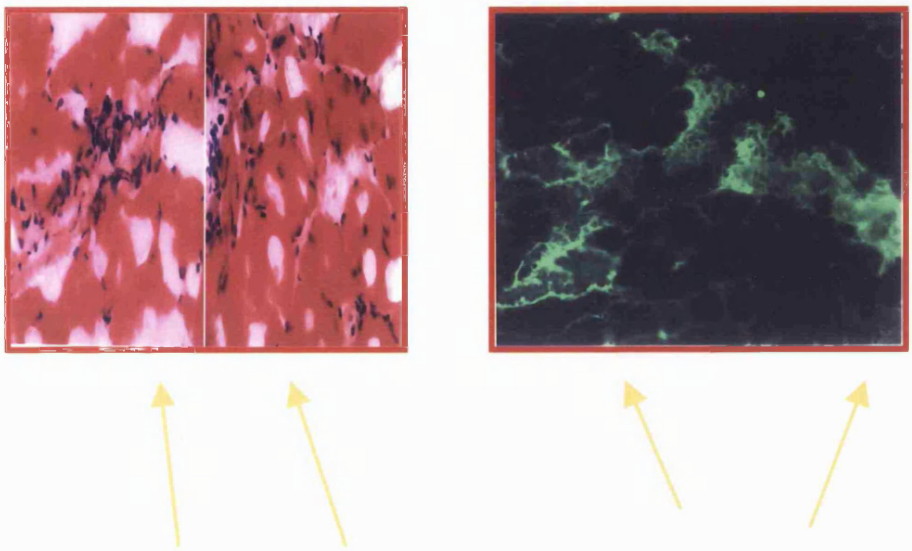
CD8 infiltrates

In the plasmid injected animals, no detectable circulating levels of factor IX could be found. As seen above, histological analysis revealed the presence of inflammatory infiltrates in the muscle sections on H&E staining. The presence of inflammatory infiltrates suggests that a cytotoxic T cell response was occurring in the plasmid injected animals. The presence of these cellular infiltrates and the strong IgG2 responses observed to the human factor IX transgene product suggested the possibility of a CTL response occurring. Further immunofluoresecent staining confirmed the inflammatory infiltrates to be composed of a high proportion of CD8 cells (*see figure 31*).

Figure 32 *Adenoviral based transduction*

The histology results for the adenoviral injected animals are shown below:

Haemotoxylin and Eosin *CD8 expression*



Inflammatory cell infiltrates CD8 expression

The adenonviral-injected animals did initially express some circulating factor IX Ag, which was detectable in the plasma as early as 2 weeks post injection (*data not shown*). However, this had disappeared by 2 weeks post injection. Therefore, a further cohort of animals was analysed at 3 weeks to study why expression was lost early. This is shown in the images above, which show the dense presence of strong inflammatory infiltrates at 3 weeks post injection. Confirmatory immunofluoresent staining confirmed the presence of CD8 positive cells amongst these infiltrates, suggesting the presence of a strong cytotoxic T cell response occurring in the muscle.

Mechanisms of intramuscular immune responses

Plasmid Injected animals

The results presented here for plasmid mediated gene transfer were disappointing. Direct plasmid injection lead to the production of profound inflammatory responses and no transgene expression. The humoral response to the transgene product displayed an immune profile in keeping with a Th1 cellular response. For non-viral

vectors such as plasmid injection of naked DNA, there is evidence to suggest that the actual plasmid backbone may act as a powerful adjuvant to the stimulation of a powerful host immune response. The basis of the immune response centres around the presence of non coding immunostimulatory sequences ("ISS") within the plasmid backbone which contain hypomethylated CpG basepairs (Sato *et al.*, 1996). These hypomethylated motifs rapidly stimulate the innate immune response with production of IFNγ by NK cells and IFN α and β, il-12 and IL-18 by macrophages.

This response is important phylogenetically as it acts as the host's first line of defence against bacterial infection. In the adaptive immune response, bacterial DNA favours the development of a Th1 driven response and secretion of IFNγ, which favours immunoglobulin class switching to the IgG2a subtype. For this reason, because of the strong and persistent cell mediated and humoral immune response to the plasmid backbone and encoded transgene, the use of plasmid DNA vaccines has enormous future potential in infectious diseases, allergy, and cancer tumour vaccines. However, for use in gene replacement expression work, to generate a secretable protein, this strong immune response is counter productive (*vide infra*). Therefore, for plasmid vectors to be effective in gene replacement strategies for secretable proteins, vectors will be needed to be designed which lack these ISS sequences. However, in DNA vaccines, these adjuvant properties would appear to be beneficial.

Adenoviral injected animals

For Adenoviral based transduction the pattern suggested by the antibody profile (IgG2) to the human factor IX suggested the presence of a cellular immune response to factor IX. Most adenoviral vectors are derived from the Ad type 5 (Ad5) virus in which a foreign expression cassette has been introduced in place of the early region 1 (E1). Such E1 deleted vectors can easily be produced at high titres in complementation cell lines, providing the E1 functions in *trans*. The deletion of the regulatory E1 genes prevents viral propagation in infected organisms and dramatically reduces the expression of all viral genes. Further deletion of the non essential early region 3 (E3) genes allow insertion of larger segments of foreign DNA (up to 8 Kb) in the viral genome. However, *in vivo* application of these E1/E3 vectors has shown that expression of the transgene is only transient, demonstrated in these experiments.

Other investigators have shown that the short lived *in vivo* expression of the transgene was related to the induction of a host specific cytotoxic T lymphocyte (CTL) immune response directed against viral antigens synthesised at low levels in the transduced tissues (Dai *et al.*, 1995), (Yang *et al.*, 1996b), (Engelhardt *et al.*, 1994a; Engelhardt *et al.*, 1994b). However, it has also been shown that cytotoxic cellular response could be elicited to the transgene itself, which could also account for the rapid elimination of transduced cells ((Juillard *et al.*, 1995), (Michou *et al.*, 1997).

Most of the early studies performed in adenoviral based transduction experiments used Beta galactosidase as the transgene. This protein is of bacterial origin and therefore theoretically could be expected to induce an immune response either cellular, humoral or both. When studied in muscle based transduction, Yang *et al.*, (1996) demonstrated that destructive cellular responses were directed to Beta galactosidase protein, i.e. the transgene product. Interestingly, however, the response was dependent on the method of gene transfer with plasmid or adenoviral vector enhancing the immune response by infecting antigen presenting cells located within the injured muscle or in the regional lymph nodes. The results shown here also show the presence of a cytotoxic cellular immune response to adenoviral and plasmid transduced muscle. It is unclear however, whether this response is directed to the transgene (i.e. human factor IX) as well as the vector. The only way to definitively answer this is to devise a CTL assay to the transgene product and study whether this phenomena occurs. This subject is discussed further in *chapter 6.*

AAV injected animals

The results from the AAV injected cohort suggest that a dominant CD4 Th2 immnue response was occuring, with a relative lack of a CD4 Th1 (CTL) response. When the work was begun with using the AAV vectors, it was hoped the expression obtained would be better than that observed in the Ad and plasmid injected animals. This was predicted, based on reports in the literature suggesting the relative lack of an immune response against these vectors, resulting in long term transgene expression. The work in one of these reports (Fisher *et al.*, 1997) demonstrated that using the *lac z* reporter gene, which produces the highly immunogenic bacterial protein ß galactosidase, long-term stable gene expression was observed for at least 32 weeks after IM injection,

suggesting the relative lack of an immune response. It is noteworthy within this study that none of the injected mice demonstrated any signs of tissue inflammation. This at the time, was a major break through since it represented the first finding of a vector which was capable of inducing long term gene expression with a relative absence of being immnunostimulatory ("blunting" of the immune response).

Subsequent reports using the AAV-lacZ or AAV-hEpo vectors showed that dose-dependent levels of β-galactosidase or human erythropoietin were obtained after single-dose administration of vector (Tripathy *et al.*, 1994), indicating an apparent lack of immunogenicity by AAV based transduction with a non-homologous transgene in this model.

Summary

From the above studies it is clear that the humoral profiles induced were influenced by vector selection. The Adenoviral and plasmid injections induce a more biased response towards a Th1 driven response whereas the AAV injected animals induce a stronger response with the production of IgG1 antibodies in keeping with a Th2 response. This would therefore, predict that the adenoviral and plasmid injected animals will produce a cellular immune response to the transduced tissue. This is borne out by the histology results, which showed evidence of inflammatory infiltration in the plasmid and adenoviral injected animals with loss of expression of human factor IX. The profile suggested by the AAV injected animals induced less of a cellular response (lack of IgG2 class induction), and this is again borne out by the histological findings of factor IX expression in the muscle sections studied. However no circulating factor IX could be detected due to the presence of a humoral response to the non species-specific transgene product (human factor IX).

5.3 Effect of Transgene

The preceding studies suggested that choice of vector alone influenced the resulting immune response, but it is clear that the expression of bacterial or human transgene

products could also constitute potential antigens and be recognised as foreign by the host immune system.

It is logical that a *non–homologous* transgene that leads to the production of a non-homologous protein will induce an innate immune response. Equally, comparison of vectors will also be influenced by the presence of transgenes coding for immunogenic proteins. Therefore, extrapolation of these findings to the homologous situation will be difficult. The subsequent cloning and availability of *homologous* transgenes facilitated more accurate interpretation of the immunological responses and allowed the following experiments to be performed with the requisite transgenes in their cognate homologous animal models.

5.3.1 Species versus Non Species specific Transgene

Aim

As outlined above, because of the observed antibody formation to the human transgene in normal immune competent mice, similar experiments were carried out in normal immune competent mice using the species specific transgene murine factor IX instead.

Method

Immunocompetent 5 month old male CD-1 mice, 5 month old male C57BL/6 mice (both strains from Charles River Breeding Laboratories, Wilmington, MA) and 5 week old male BALB/c mice (The Jackson Laboratory) (n=3 for each strain) were used in this study. The quadriceps and tibialis anterior muscles of both hind limbs were injected with a total dose of $1x10^{11}$ AAV-mFIX. The positive control mice used for these experiments was injection of the same AAVmFIX construct into knockout haemophilia B mice.

AAV mFIX Vector

AAV vector construction.

The plasmid encoding AAV-2 vector AAV-CMV-mFIX, contained the murine FIX cDNA (2.7-Kb *Bam* HI fragment) under the transcriptional control of a cytomegalovirus IE enhancer/promoter, and also included a chimeric CMV/β-globin mini-intron (5' to the mFIX cDNA), and the human growth hormone polyadenylation signal. The expression cassette is flanked by AAV serotype-2 inverted terminal repeats (ITRs). AAV-CMV-mFIX was produced by triple transfection of HEK-293 cells in a helper virus-free system, and purified by repeated CsCl gradient centrifugation (as described in chapter 4, page 110). Vector titres were quantitated by slot blot hybridisation. Purified vector was stored at –80°C in PBS containing an osmotic stabiliser, and further diluted with sterile PBS prior to injection of the mice.

Antibody detection

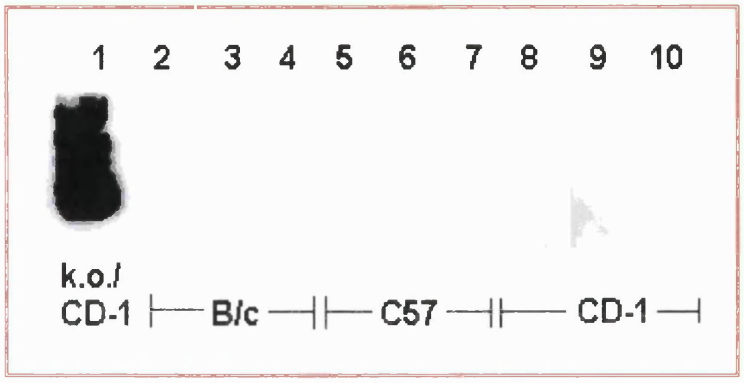
The presence of antibodies was detected by Western blot. Murine factor IX was transferred onto a nitrocellulose membrane and incubated with various mouse plasma samples, followed by incubation with horseradish peroxidase conjugate anti-mouse IgG and autoradiographic detection.

Results

Anti mFIX antibody detection

None of the mice injected with AAV-mFIX developed antibody against murine factor IX when assayed by Western blot at 18 days (data not shown) and 60 days post-injection (*Figure 33*, lanes 2-10).

Figure 33 *Western Blot Normal mice with AAVmFIX transgene*



Lane 1. *Plasma from haemophilia B mouse bred on CD-1 background that had developed antibodies against human factor IX after intravenous injection of Ad-hFIX. These antibodies cross-reacted with murine factor IX.* ***Lanes 2-10.*** *Plasma from mice injected IM with AAV-mFIX.* ***Lanes 2-4.*** *BALB/c mice.* ***Lanes 5-7.*** *C57BL/6 mice.* ***Lanes 8-10.*** *CD-1 mice. All samples were from day 60 post-injection.*

Clotting times by APTT on plasma samples of all vector injected mice were within the normal range (approximately 25 seconds) when measured 60 days post-injection. Factor IX inhibitor assays on all the injected mice also demonstrated the absence of Factor IX inhibitors. The control mice injected with AAV-mFIX all developed antibodies against murine factor IX within the first 4 weeks of injection, demonstrating the immunocompetence of these animals.

Summary

Although an innate expectation, the critical importance of these studies demonstrates that normal immune competent mice do not make antibodies to the transgene product murine factor IX. This is in contrast to using human factor IX as the transgene, where all injected animals made antibodies to the transgene product. These studies emphasise the importance of selecting a *species-specific transgene* in any proposed gene transfer studies.

The majority, if not all of the early gene therapy studies performed, employed the use of non-species specific transgenes (usually human). This clearly made interpretation

of the results difficult, since immune responses (as would be predicted) frequently occurred. The design of future gene therapy experiments needs to consider this, and emphasises the importance of selection of the relevant homologous transgene, and that when planning gene transfer strategies the choice of transgene is critical.

5.4 Other Factors That May Affect the Immune Response

5.4.1 Route of Administration of the vector

The route of administration of antigen delivery clearly influences the outcome of immune responses. In a gene-based approach, an important element of how much gene expression takes place will depend on the target tissue reached and the immunological cell populations present. For example, tissues rich in antigen presenting cells (APCs) may effectively induce immune responses more than tissues not rich in APCs.

It is known that individual immunogens exhibit particular dose response curves, which may be determined by measuring the immune response observed with various doses and administered route of delivery. An insufficient dose does not lead to immunogenic stimulation, either because it fails to activate sufficient lymphocytes or because it induces a non-responsive state. Conversely, an excessively high dose can also fail to induce a response because it can cause lymphocytes to enter a non-responsive state. In mice, the immune response to purified pneumoccal capsular polysaccharide illustrates the importance of dose. A dose of 0.5mg fails to induce an immune response in mice, whereas a thousand fold lower dose (5×10^{-4} mg) induces a humoral antibody response (*Kuby J*, Immunology, (WH Freeman, 2000) pages91-92). A single dose of most experimental immunogens will not induce a strong response. Rather, repeated administration, over a period of weeks is required to stimulate a strong response. Such repeated administrations or boosters increase the clonal proliferation of antigen specific T or B cells.

Experimental immunogens are generally administered parentally, that is by routes other than the digestive tract. The following routes of administration are common:

- *Intravenous*
- *Intradermal*
- *Subcutaneous*
- *Intramuscular*
- *Intraperitoneal*

The administration route determines which immune organs and cell populations will be involved in the response. Antigen administered intravenously is a very effective route to the spleen because the blood vessels open into this organ and antigen is effectively filtered from the blood. Antigen administered subcutaneously and intramuscularly moves first to the local lymph nodes but may also reach the spleen by lymph flow and the thoracic duct. Differences in the lymphoid cells populating these organs generate differences in the quality of the subsequent immune response. Therefore the route of administration is critical to the outcome of an immune response to a particular immunogen, and variation in route will influence outcome.

In the conventional protein based replacement therapy for haemophilia, infusion of clotting factor concentrates results in initial presentation in the spleen and antigen presentation occurs at this site. However, when antigen delivery is via a peripheral route such intramuscular or subcutaneous injection antigen presentation occurs via antigen presenting cells in regional draining lymph nodes. In a gene based approach the target tissue selected will influence outcome, as the draining lymph nodes of that particular tissue will first present antigen to the immune system. In the case of an intramuscular approach in the hindlimbs of a mouse, the draining lymph nodes will be the inguinal and popliteal nodes of the injected limb.

Route of Administration and Gene Transfer

Introduction

It has been observed by a number of investigators (Walter *et al.*, 1996; Michou *et al.*, 1997), that when an adenoviral vector expressing a non homologous transgene, such as human factor IX is injected intravenously into C57BL6 mice, a failure of antibody production is observed, with high circulating levels of human factor IX antigen being produced (Connelly & Kaleko, 1998), (Kung *et al.*, 1998). This seems a rather unexpected result given the high immunogenicity of Adenoviral vectors (Yang *et al.*, 1994a; Yang *et al.*, 1994b; Yang *et al.*, 1994c) and the use of a non-species specific transgene in the vector.

If the same injection is performed intramuscularly in the same strain of mice, no circulating FIX antigen is found in the serum of the injected animals and all the animals make antibodies to the human factor transgene product, which is predominantly an IgG2 subclass. Close analysis of the injected muscle reveals that although expression of factor IX can be demonstrated at a tissue level for a transient time (2-3 weeks), there is a gradual build up of inflammatory infiltrates in the muscle precluding further expression of the transgene and the ultimate destruction of the target tissue by a cytotoxic T cell infiltrate (as noted in the histological sections shown above, *see page 123*).

It is puzzling therefore, why the introduction of a human transgene product should result in long-term expression in normal and in knock out mice that are deficient in F.VIII or FIX using a vector that is well-characterised for its immunogenicity (Yang *et al.*, 1994a; Yang *et al.*, 1994b; Yang *et al.*, 1996a; Yang *et al.*, 1996d). Studies have shown that haemophilic C57BL/6 mice are not tolerant to human coagulation factors, since they rapidly develop high titre antibodies against human FVIII or FIX when these proteins are introduced as intravenously infused protein concentrates or when the vector is injected intramuscularly (IM)(Qian *et al.*, 2000).It is therefore unclear why long-term expression of coagulation factor IX is achieved in C57BL/6 following systemic delivery of adenoviral vector, and how the intravenous route of administration fails to result in production of antibodies in the C57BL6 strain. Other investigators have commented on the ability of the C57BL6 to demonstrate prolonged

expression with other transgenes, such as human antitrypsin, (Barr *et al.*, 1995), when compared with other strains of mice.

5.4.2 Experimental strategies to address route of administration

The aim of the studies in this section was designed to examine the influence of route gene delivery in the C57BL6 strain and, examine the mechanisms, which may be involved in the paradoxical immune responses observed in the C57BL6 strain of animals. The experimental strategy taken was to compare the effects of route of administration in two different wild type strains of mice (C57BL6, Balb/C).

Method

4-6 week old male inbred C57BL6 and Balb/C mice (n=4) were purchased from the Jackson Laboratories (Bar Harbour, ME, USA) and injected intravenously via the tail vein with an Adeno viral vector encoding human factor IX. The mice were bled at days 0, 7, and 14 days after injection and bi weekly thereafter.

Vectors

The vectors used for these experiments contained an expression cassette encoding the human FIX cDNA under the control of a CMV IE enhancer/promoter. The vectors used for these experiments were produced using the methodology described previously (Chapter 4, page 106). The dose of vector injected was $4x10^{10}$ vector genomes per animal.

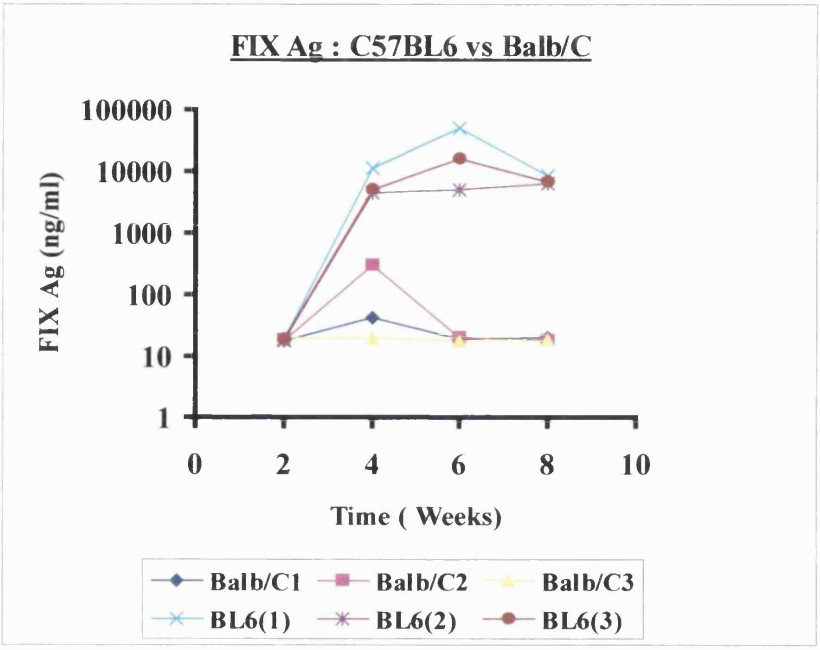
Human FIX antigen and anti human factor IX measurement

Human FIX antigen in plasma samples of mice was measured by Elisa as described (Chapter3, page 66). This assay does not detect murine FIX. Antibodies against human FIX were detected in plasma samples (1:32 dilution) by subclass specific Elisa as described. (Chapter3, page 67).

Results

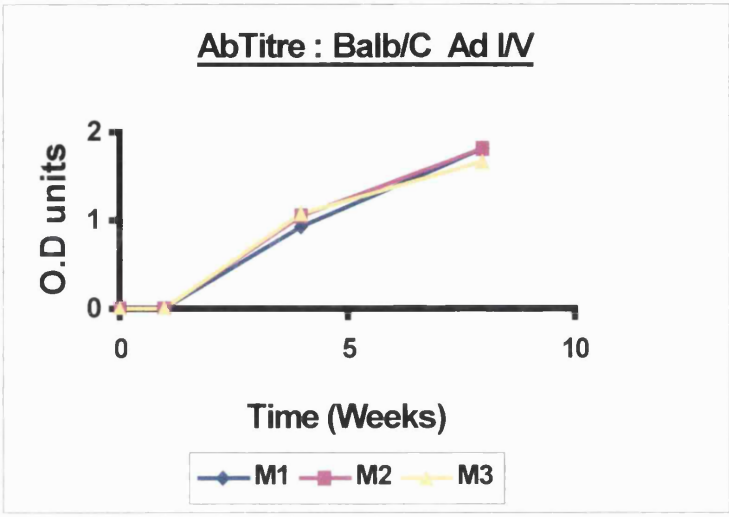
The figures below show Factor IX antigen expression and anti FIX antibody production in C57BL6 and Balb/C miceachieved after intravenous injection (tail vein) of an adeno virus encoding human FIX into wild type Balb/C or C57BL6 mice.

Figure 34 *FIX Ag expression in WT C57BL6 and Balb/C mice*



As demonstrated from the above graph, expression reached in the Balb/C mice is very low and lost quickly in this strain. However, in the in the C57BL6 mice the expression levels reached are logarithmically higher and sustained. When analysed at much later time points, the C57BL6 mice continue to express high amounts of circulating FIX antigen. (*data not shown*)

Figure 35 *Antibody titres Balb/C mice*



The presence of antibodies is easily demonstrated in the Balb/C animals serum, but not in the C57BL6 strain (*data not shown),* thus reflecting the antigen levels obtained.

Summary

These results demonstrate that these two strains respond significantly differently when challenged by gene transfer when vector administration is via the same route. The inference from these results is that ***strain specificity*** directly influences the response to gene transfer. These results pose the question as to why therefore the C57BL6 strain is permissive (and produces large amounts of FIX antigen) to a highly immunogenic challenge with Adenoviral human factor IX when administered intravenously compared to Balb/C mice. The following experiments were conducted to answer some of these questions.

Are the C57BL6 injected mice truly tolerised?

Aim

As shown above, the C57BL6 injected mice continue to express large amounts of factor IX and suggesting that these mice may truly be tolerant to the circulating human factor IX. If this were the case, it might be expected that further

immunological challenge to these animals, with human FIX, would result in continued expression of circulating factor IX. This hypothesis was tested out in the following experiment.

Method

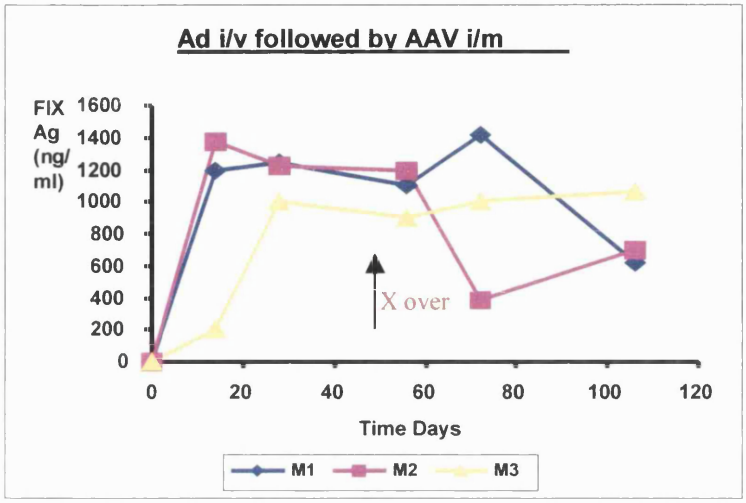
A cohort of mice, who initially received Adenovirus encoding hFIX IV, were later challenged (8 weeks) with an AAV vector IM encoding human factor IX. Normally immune competent mice receiving AAV IM injections produce an antibody to factor IX that results in no circulating FIX levels being detectable. If the mice injected with Adenovirus encoding factor IX were truly tolerised, then later challenge with the AAV IM injection should not result in the production of antibodies to factor IX after injection.

At day 0, four C57BL6 mice received a dose of AdhFIX at a dose of $4x10^{10}$ vector genomes via tail vein injection. The mice were then analysed for factor IX antigen and antibody production by the methods previously described. (Chapter 3, page 65). At 8 weeks (cross over point) the mice then received AAVhFIX at a dose of $4x10^{10}$ vector genomes given intramuscularly into the hindlimbs. The mice were again followed up serially at 2 weekly intervals post injection for FIX Ag and antibody formation to circulating human factor IX.

Results

The following figure shows the antigen levels before and after the mice received the AAV intramuscular injection

Figure 36 *Adenovirus IV followed by AAV I/M*



As demonstrated from the above results, these mice continued to produce significant amounts of circulating factor IX and did not drop their levels after the AAV I/M injection suggesting that the animals were truly *tolerant* to the circulating human factor IX.

Further confirmation of tolerance induction:

Lack of hFIX-specific T cell proliferation in C57BL/6 mice that had received IV injection of adenoviral vector.

C57BL/6 mice (n=3) that had received adenoviral vector via the IV route were challenged twice by IP injection of hFIX protein formulated in complete Freund's adjuvant (cFA) 5-6 months after adenoviral vector administration. The injections were one month apart, and the animals did not show antihFIX as measured by ELISA, up to two weeks after the last boost with hFIX/cFA (data not shown). Subsequently, the mice were sacrificed and *in vitro* proliferation of lymphocytes in response to hFIX antigen was assessed. C57BL/6 that had received IM injection of the adenoviral

vector (but not cFA) were analyzed in parallel. As documented in the table below no evidence for T lymphocyte proliferation was obtained for mice that were IV injected with adenoviral vector, despite the repeated boost with hFIX antigen in cFA, while IM injected mice showed mild proliferation with a stimulation index of 2-3.5.

Table 5 *Mouse Stimulation indexes*

Mouse	SI
Ad-hFIX IM	2.1
Ad-hFIX IM	3.5
AAV-hFIX IM + Ad-hFIX IV + hFIX/cFA	<1
Ad-hFIX IV + AAV-hFIX IM + hFIX/cFA	<1
Ad-hFIX IV + AAV-hFIX IM + hFIX/cFA	1.6

Lymphocyte proliferation following in vitro stimulation with human FIX protein. Shown are the stimulation indexes (SI) for individual C57BL/6 mice that had received ad-hFIX vector IM (first two rows) or IV (last three rows). Mice injected IV with Ad-hFIX vector had received IM infusions of AAV-hFIX 8 weeks before or after IV administration of the adenoviral vector, and were boosted twice in a 1month interval with hFIX protein formulated in complete Freund's adjuvant (cFA) and infused intraperitoneal (IP). SI was calculated based on the hFIX concentration that gave the highest proliferative response, which was generally, 5-10 μg hFIX/ml medium.

Summary

The failure of the splenocytes, in the intravenously injected animals, to proliferate to the potent resimulation challenge of human factor IX in complete Freunds adjuvant indicates tolerance has been induced to hFIX in these animals. The mechanisms of why this occurred is outlined in thefollowing section.

Possible mechanisms to explain the observed C57BL6 tolerance

If the C57BL6 strain is truly tolerant to circulating factor IX Ag (after intravenous administration), it may be that this antigen is inefficiently presented to the effector

immune system and result in T cell anergy. The failure of efficient presentation can occur for several reasons in the antigen-presenting pathway. The APCs may carry insufficient co stimulatory molecules; or they may lack the required MHC class II molecules to present antigen to effector CD4 cells.

Charlton (Charlton *et al.*, 1997) studied the ability of splenic antigen presenting cells, from nine independent mouse major histocompatibilty complex (MHC) haplotypes, to present recombinant streptoccocal exotoxin A (rSPEA) to heterogeneous T cells and mouse T cell clones using using proliferation assays.

The authors reported a marked variation between in proliferative capacity between MHC haplotypes, which were ranked as follows:

$H2^{z} > H2^{s} = H2^{f} = H2^{p} = H2^{r} = H2^{k} > H2^{d} > H2^{b} = H2^{q}$

($H2^{b}$, C57BL6 strain)

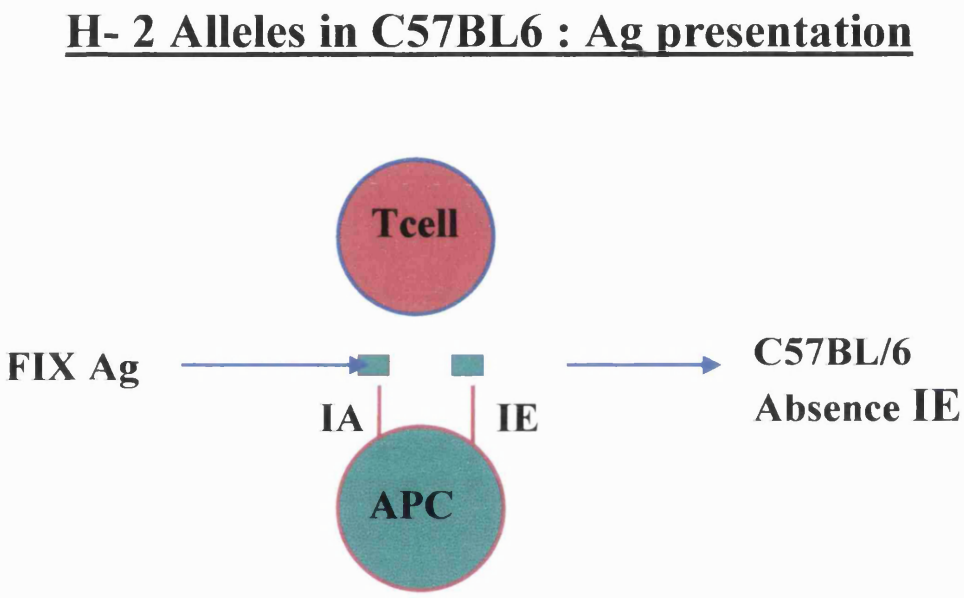
The conclusion from these studies was that there was significant inter strain variation in the ability of different alleles of both E and A molecules (these are the homologous murine equivalent to class II molecules in man) to bind and present rSPEA.

5.5 Role of Haplotype in FIX Presentation

From the work described above, it was shown that haplotype influences antigen presentation, and the likely occurrence of immune responses (Charlton *et al.*, 1997). In the following series of experiments the role of haplotype was examined to determine whether the haplotype of the C57BL6 strain was playing a role in the observed responses to intravenous administration (Fields *et al.*, 2001a) of Adeno viral vectors. C57BL/6 is an inbred strain of mice (haplotype $H\text{-}2^{b}$) that is deficient in the MHC class II allele IE, but can present peptides by MHC class II molecules encoded by the IA allele.

In particular, the question asked was whether the absence of IE in C57BL6 was affecting the response to circulating factor IX after AdhFIX IV administration. Since C57BL6 mice are deficient in the class II allele IE, it could be hypothesised that this allele is important in antigen presentation of human factor IX as shown below:

Figure 37 *Role of H-2 Alleles in antigen presentation*



As can be seen from the above diagram, if the presence of the **IE** allele is required for antigen presentation, no factor IX antigen may be presented to reactive effector T cells, resulting in failure of induction of an immune response (via T cell ignorance) and resulting factor IX expression.

Aim

This hypothesis was tested by conducting a similar experiment in another strain of mouse also deficient in the class IE allele. If the absence of IE is truly implicated, elevated levels of factor IX should circulate in this haploidentical strain (IE deficient)

Some commonly listed mouse haplotypes are listed in the figure below:

Table 6 *Murine Haplotypes*

H2 HAPLOTYPES OF COMMON MOUSE STRAINS

Strains	Haplotypes	H-2 gene complex
		K A E S D
C57BL6 C57BL10, FVB	b	b b - b b
BALB/c , DBA/2	d	d d d d d
AKR,CBA,C3H	k	k k k k k
SWR	q	q q q q q

A mouse strain with a similar haplotype to the C57BL6 mouse is the FVB strain i.e. IE deficient. Therefore, a similar experiment was performed in this strain, whereby intravenous AdhFIX was administered to a cohort of 4 FVB mice.

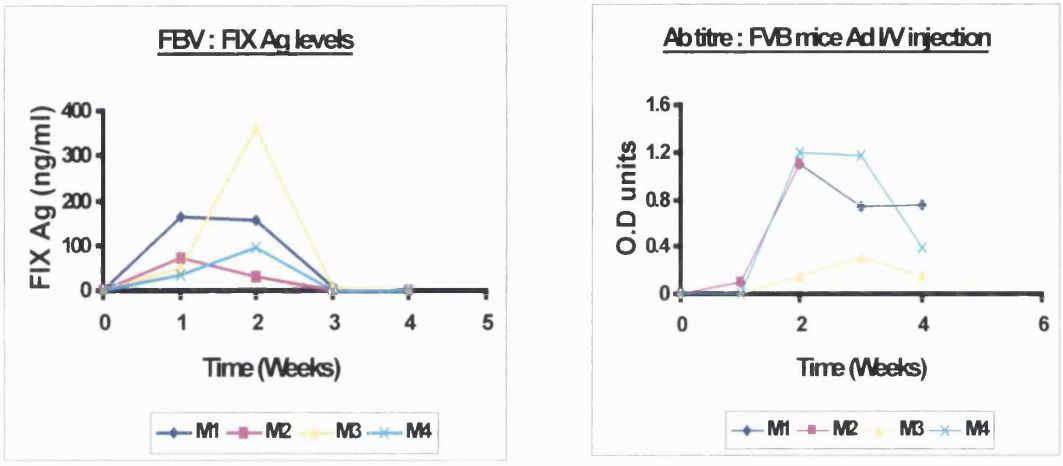
Method

A cohort of four *FVB* mice (n=4 obtained from Charles River Laboratories USA) were injected intravenously at day 0 with AdhFIX vector at a dose of 4x10 vg per animal.

Vector

The vector selected was an Adeno viral vector encoding the human factor transgene under the control of a CMV promoter. The dose of vector administered was $4x10^{10}$ vector genomes per animal.

Figure 38 *Antigen and Antibody levels in FVB following AdhFIX injection*



Ag **Ab**

The right hand panel in the above graph also illustrates that the falling levels of factor IX antigen coincided with the appearance of circulating antibodies to the factor IX antigen, indicating that the situation seen in these animals was not that echoed in the haploidentical C57BL6 strain. Therefore the lack, of the IE allele does not appear to be directly responsible for the C57BL6 unique response to AdhFIX intravenous injection. This result appears to be in keeping with another group`s attempts to explain the long term gene expression observed with the C57BL6 strain using the alpha antitrypsin transgene (Barr *et al.*, 1995).

5.6 The Role of Interleukin 10

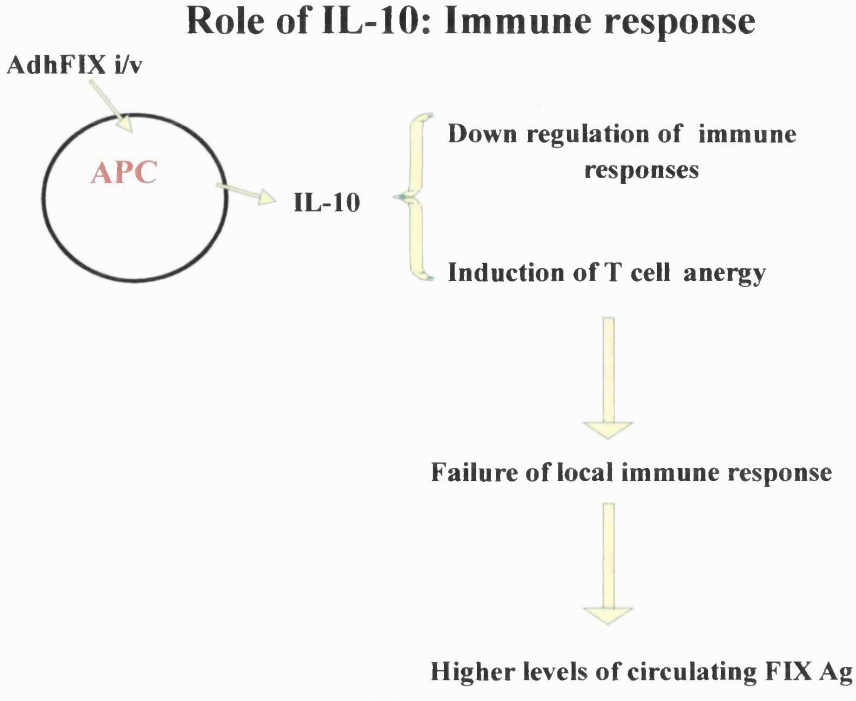
An alternative explanation for tolerance induction in the C57BL/6 may be the unique pattern of cytokine expression following systemic delivery of the viral vector via the intravenous route. Interleukin 10 (IL-10) has been documented to be involved in antigen-specific tolerance by its ability to down-regulate immune responses, including

down-regulation of MHC class II expression and inhibition of production of inflammatory cytokines (Tan *et al.*, 1995). IL-10 is implicated in the negative regulation of T cells and induction of tolerance in $CD4^+$ T cells (Knolle *et al.*, 1995; Knolle *et al.*, 1998a; Knolle *et al.*, 1998b). Expression of IL-10 in Kupffer cells, professional APCs (dendritic cells) in the liver, may serve as an autoregulatory mechanism for antigen presentation by liver antigen presenting cells (Knolle *et al.*, 1998b). It has been shown that Kupffer cells secrete IL-10 in response to endotoxin challenge and regulate local immune and inflammatory reactions in the liver sinusoid (Knolle *et al.*, 1995).

Aim

In the intravenous route of administration the majority of antigen presentation takes place in the liver. In order to examine whether regulation of IL-10 expression in the liver, the main target of gene transfer following IV injection of adenoviral vector, plays a role in tolerance or immunity against FIX antigen, two sets of experiments were carried out, both in wild type C57BL6 and Balb/ C and also simultaneously in the same strains deficient for IL-10. The aim of this experiment was to test out whether IL-10 was playing a role in the observed tolerance to the circulating factor IX antigen in the C57BL6 animals. The central hypothesis being tested is outlined schematically below:

Figure 39 *Role of IL-10*



Methods

2 cohorts of IL-10 deficient mice (C57BL6 IL-10 KO, Balb/C IL-10 KO) were challenged with IV (via tail vein) injections of the adenoviral vector encoding hFIX. The IL-10 knockout (KO) mice were provided by Professor J Farrell at the University of Pennsylvania Veterinary School.

Vectors

The vector used for these experiments was an E1/E3-deleted adenoviral vector, ad-hFIX expressing of human FIX from the CMV enhancer/promoter was constructed as described (Walter *et al.*, 1996). The vector was replicated in HEK-293 cells and purified from cell lysate by two rounds of CsCl density gradient centrifugation using standard protocols. The vector has a total particle: pfu ratio of 25:1 as determined by plaque assay (Walter *et al.*, 1996).

FIX Antigen and Antibody estimation

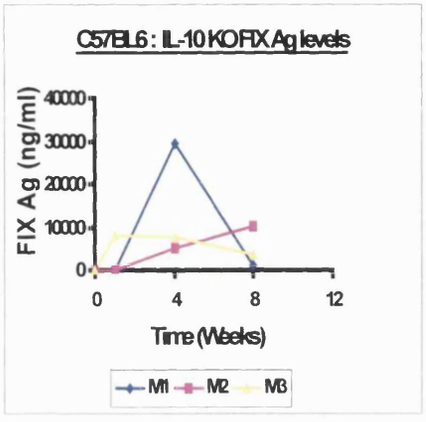
Human FIX antigen in murine plasma samples was measured by Elisa as described. (Chapter 3, page 65). This assay does not detect murine FIX. Antibodies against human FIX were detected in plasma samples (1:32 dilution) by subclass specific Elisa as described (Chapter 3, page 66).

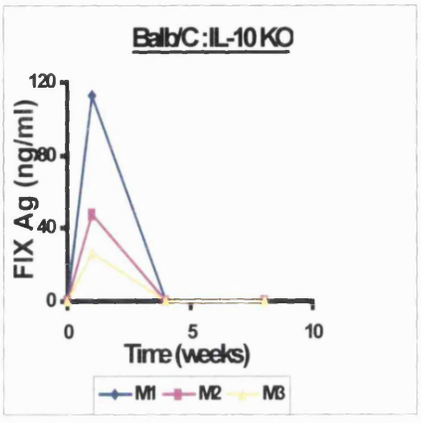
Results

The graph below shows the antigen levels achieved in the IL-10 knockout animals. BALB/c mice (haplotype H-2^K) have been shown by others to produce anti-hFIX following IV injection of adenoviral vector (Michou *et al.*, 1997). As shown in the graphs below, IL-10 deficient C57BL/6 mice produce high systemic levels of hFIX without detectable antibody formation.

Figure 40 *FIX Ag IL-10 KO mice*

FIX Ag levels : IL-10 KO mice

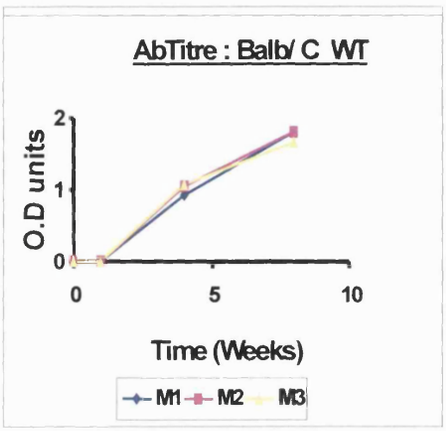


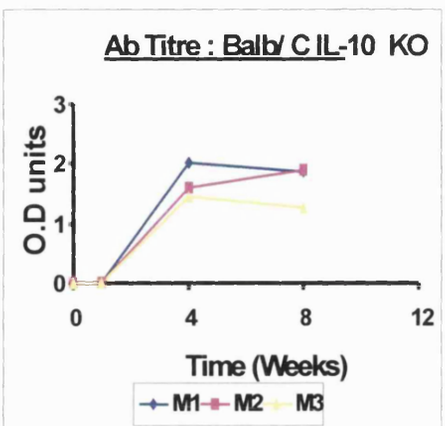


The IL-10 knockout Balb/C mice failed to produce high level and long-lived antigen expression. This would be expected if IL-10 was truly acting as an immunosuppressor in liver derived antigen presentation of human factor IX.In contrast to C57BL6 mice, normal and IL-10 deficient BALB/c mice synthesised IgG1, IgG2a, and IgG2b anti-hFIX by day 14 post vector administration, thereby blocking systemic expression efficiently. This is illustrated in the figures below:

Figure 41 *Anti FIX Ab Balb/C mice*

Anti FIX Ab : Balb/c mice





It therefore appears, from the data described above, that there is no evidence of involvement of IL-10 expression in the tolerance observed to hFIX to explain the high levels of factor IX expression observed in the C57BL6 strain via the intravenous route.

Summary

The previous two sets of experiments (role of haplotype and role of IL-10) attempted to address the possible mechanisms of the paradoxical results displayed by the C57BL6 strain of mice. Neither hypothesis could explain the results observed. The results displayed by the C57BL6 strain point out the importance of *strain variability* and their responses to gene transfer (Fields *et al.*, 2001a). This point should be borne in mind when devising pre-clinical gene therapy protocols. Therefore, because of their inherent permissive state, interpretation of gene expression in this strain may not be relevant when extrapolating to other strains of mice and different species of animals.

Discussion

When considering the immune responses to gene transfer there are many factors to take into account, both internal and external to the process, which may influence the outcome. The early part of this chapter showed that vector selection plays a significant role in the outcome and leads to the generation of differing immune responses according to which vector is selected. (Adenoviral, Plasmid > Adeno associated). Therefore, the choice of vector is crucial to the overall efficiency of the procedure and this point clearly needs to be borne in mind when designing gene therapy protocols in animal models and humans. In this respect, it appears that AAV viral vectors appear to induce a T helper cell response that results in a dominant IgG1 subtype antibody to human factor IX.

Based on the observations from immunoglobulin profile data, it may be predicted that AAV muscle based transduction induces a more dominant Th2 response, which is associated with a less cellular cytotoxic T cell response as compared to the plasmid and adeno viral responses. The only definitive way to confirm these observations is to develop a Cytotoxic T cell assay (CTL) to the transgene product, human factor IX. This is the subject of the work discussed in the following chapter.

Similarly, the choice of transgene influences outcome. Early experiments in gene-based strategies were hampered by the lack of appropriate homologous transgenes available for study in the relevant animal models. This has not allowed straightforward interpretation of the immune responses generated. Now, with the availability of the relevant homologous transgenes and the wider availability of vector choice, a more critical evaluation of immune responses may be carried out in animal model preclinical studies. Finally another important consideration to take into account is the effect of strain on the results obtained. Many early studies in gene therapy used the C57BL6 strain as the recipient animals of gene transfer. Bearing in mind their relative permissive state to high immunogenic challenge, the results obtained for this strain should be born in mind with relative caution.

The results also point out that, in the wider context of gene therapy, for each different gene therapy strategy planned (i.e. a particular combination of vector, transgene and target tissue), the immune response needs to be critically evaluated for each individually designed component of the protocol. Finally, other factors, such as route of administration of gene delivery clearly influence outcome and also need to be taken into account when devising gene therapy protocols.

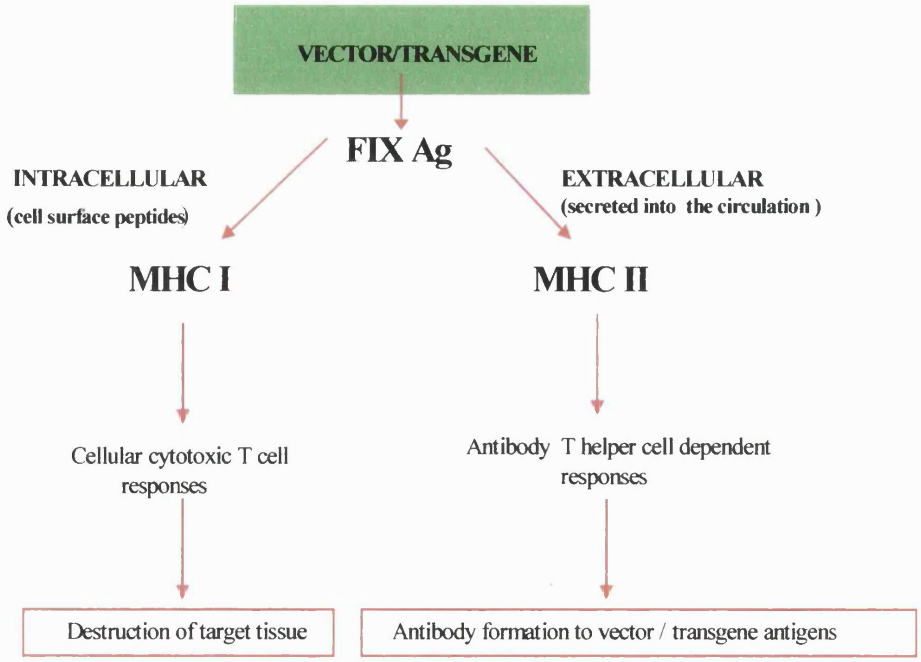
CHAPTER 6

CELLULAR BASED IMMUNE RESPONSES IN NORMAL AND HAEMOPHILIC MICE

6.1 Introduction

Compared to conventional treatment of haemophilia by the infusion of plasma derived or recombinant clotting factor concentrates, a gene-based approach involves both the intracellular and extracellular production of clotting factor protein. This approach therefore, will involve different mechanisms of antigen presentation. Generally, immune responses in a gene therapy setting may differ from those encountered during infusion of protein because the endogenously synthesised FIX is presented in context of MHC class I as well in addition to MHC class II determinants. A transgene product expressed by cells following gene transfer, presented by MHC class I molecules on the cell surface, will create a potential target for destruction by MHC class I-restricted cytotoxic T lymphocytes (CTLs, $CD8^+$ T cells). The differences in the pathways are shown below and illustrate the possibility of entering an MHC I pathway in addition to the generation of an MHC II pathway. Therefore in a gene-based approach consideration needs to be given to the possibility of an MHC class I response occurring leading to the generation of a cytotoxic T cell response.

Antigen processing : Presentation in a gene based approach



the non-species-specific transgene product hFIX which are predominantly composed of an IgG1 subtype (chapter 5, page 120).

This observation suggests that a cellular cytotoxic T cell response may not be occurring in the context of AAV muscle directed gene transfer. In order to prove whether this is true, the only definitive way is to set up an assay which is capable of showing whether a cytotoxic T cell response (CTL) is induced to human factor IX. At the time of starting this work no published study had addressed this question.

Aim

The aim of the studies in this chapter was to see whether AAV vectors encoding the human factor IX transgene could elicit a cytotoxic T cell response to factor IX antigen in a muscle directed gene transfer approach.

6.2 Development of Cytotoxic T Cell Assays (CTL)

The development of a cytotoxic T cell assay requires many stages and reagents. The development of the assay was first tested by using the reporter gene Beta Galactosidase and using the protocol as previously published (Fisher *et al.*, 1997). Once efficacy was demonstrated with the reporter gene, the assay was refined to test out the CTL response against the secretable protein human factor IX.

6.2.1 CTL responses to ß galactosidase protein

Before embarking on assays to test the CTL response to a secretable protein i.e. factor IX, a CTL assay was set up to reproduce the previously published data about the CTL response to the beta galactosidase protein encoded by an Adeno associated vector and an adenoviral vector. The previously suggested data reported there was a strong CTL response to the Beta galactosidase protein encoded by an adenoviral vector (Fisher *et al.*, 1997) but not by an Adeno associated vector. It should be borne in mind that Beta galactosidase protein is a highly immunogenic non-secretable bacterial protein and the results yielded by this protein may differ from the human secretable transgene product human factor IX.

Two processing pathways

The diagram above illustrates that there are two possible antigen-presenting pathways. Intracellular (endogenous) and extracellular (exogenous) antigens present different challenges to the effector immune system. Extracellular antigens such as infused clotting factor concentrates are eliminated by secreted antibody, whereas intracellular antigens i.e. those produced endogenously are most effectively eliminated by cytotoxic T lymphocytes. To mediate these responses the immune system uses two different antigen-presenting pathways; Endogenous antigens are processed in the cytosolic pathway and are presented on the membrane with MHC class I molecules. Exogenous antigens such as infused soluble protein antigens are presented on the membrane with class II MHC molecules.

Gene transfer to professional antigen presenting cells (APCs) such as dendritic cells may result in MHC I presentation and, subsequently, activation of antigen-specific CTLs (Yang *et al.*, 1995a; Yang & Wilson, 1995; Yang *et al.*, 1995c; Jooss *et al.*, 1996; Jooss *et al.*, 1998a; Jooss *et al.*, 1998b; Jooss *et al.*, 1998c). Uptake of exogenous protein by APCs will result in presentation of peptides by MHC class II molecules, thereby potentially activating T helper cells ($CD4^+$ cells). In mice, cells of the T helper cell subset are known to differentiate into Th1 or Th2 cells depending on factors such as the local cytokine milieu. Proliferating Th2 cells characteristically secrete IL-4 and IL-10 cytokines and are capable of activating B cells resulting in mice in production of antibody dominated by the IgG1 isotype. Th1 cells, characterised by secretion of IL-2 and IFN-γ, stimulate proliferation of CTLs, and activate B cells secreting IgG2a (Yang *et al.*, 1995a; Yang & Wilson, 1995).

While Th2/IgG1 responses are typical for the immune response against an infused soluble protein, Th1/IgG2a responses are more typical for protein expression in the context of viral infections (Yang *et al.*, 1994b; Yang *et al.*, 1995b; Yang *et al.*, 1995c; Yang *et al.*, 1996a). However, depending on the nature of the protein, dose, route of administration, and presence of adjuvant, an infused protein may also cause Th1 responses (Lagrange, 1977). As shown in chapter 5 in response to AAV based transduction, expression of human FIX in immunocompetent mice persists in AAV vector-transduced muscle fibres despite the presence of neutralising antibodies against

Method

The experiment was performed by injecting two cohorts of four mice intramuscularly (C57BL6 littermates) either with an adenoviral or adeno associated vector encoding β galactosidase at a dose of 4 $x10^{10}$ vector genomes per Kg. At day 10 a CTL was performed to assess the cytotoxicity of the recipient lymphocytes against a target cell expressing the β galactosidase protein.

Generation of Target cells

Briefly target cells (MHC-1 compatible, C57SV cells: a gift from Dr Yvonne Patterson, University of Pennsylvania) were mock infected, infected with Adlac Z, or stably transduced with a *lac Z* retrovirus.

Generation of Effector cells

At day 10 the spleen and draining lymph node cells of the injected animals were removed and cell suspensions prepared to act as effector cells. These cells were restimulated *in vitro* for 5 days with β galactosidase protein (10 μg/well, Sigma), AAV lac z, or AD lacz ($5x10^9$ /well, UV inactivated), or mock medium in 24 well plates. At the end of the stimulation period conventional chromium release assay was performed as previously described (Yang *et al.*, 1996a).

Results

a) In vitro Cytolysis of ß galactosidase

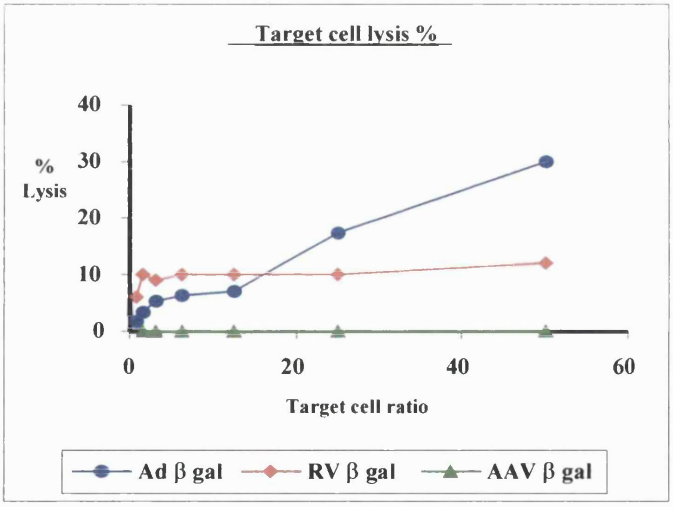
The results for the degree of cell lysis are shown in tabular form below: As can be seen maximal cell lysis occurs for those target cells labelled with Adβgal. Lysis also occurs to the RVβgal labelled cells. The higher degree of lysis seen in the former represents a CTL to both the Adenovirus and the β gal protein components of the Adβgal vector.

	%LYSIS		
TC Ratio	Ad ß gal	RV ß gal	AAV ß gal
50	30	12	<1
25	17.4	10	<1
12.5	7	10	<1
6.25	6.4	10	<1
3.12	5.4	9	<1
1.5	3.2	10	<1
0.75	1.8	6	<1

b) AAV vs. Ad: Cytolysis for ß galactosidase

The graph (*figure 42)* below shows that the Adenoviral-immunised animals exhibit a strong cytotoxic response against the target cells B galactosidase (30-40% lysis target cells). In contrast the animals immunised with an adeno-associated vector failed to demonstrate a significant CTL response.

Figure 42 *% Target cell lysis Beta Galactosidase*



Summary

From the above experiment it appears that the AAV vector did fail to induce a CTL response when encoding the non-secretable transgene B galactosidase. The data from these experiments were further supported by analysis of the mouse immunoglobulin subtypes to B galactosidase in the immunised animals. It was shown that the dominant subclass induced in the adenoviral-immunised animals was of a IgG2 subclass,

whereas in the adeno-associated immunised animals the dominant subclass induced was an IgG1 subclass. These profiles would reflect Th1 driven cellular CD4 response for the adenoviral immunised animals and a more dominant Th2 response in the adeno associated immunised animals. The next stage of the experiments was to design a study to determine whether there were cytotoxic T cell responses to the secretable transgene product human factor IX.

6.3 A Study of The CTL Responses to the Transgene Product Human Factor IX in the Context of Adeno-Associated or Adenoviral Mediated Gene Transfer

In order to address the question of whether a cytotoxic T lymphocytes (CTL) response was occurring against the secretable transgene product human factor IX, an *in vitro* CTL assay was set up.

Experimental design

These experiments were set up to determine if normal mice (C57BL6) immunised with an Adeno associated viral vector encoding human factor IX could generate a cytotoxic T cell response to human factor IX. The experiments were also performed in haemophilic mice on a C57BL6 strain background. As a positive control, separate cohorts of mice were also immunised with an adenoviral vector encoding human factor IX. The methods for the CTL assay were developed along the same lines as those previously described (Yang *et al.*, 1996b; Fisher *et al.*, 1997; Jooss *et al.*, 1998a).

Generation of antigen stimulated T cells (Effector cells)

C57BL/6 mice were immunised by IM injection with adenoviral or AAV vectors expressing hFIX at a dose of 4 $x10^{10}$ vector genomes per Kg (n=3 per vector). The identical experiment was also carried out with two cohorts of haemophilia B mice (C57BL/6 background) immunised with ad or AAV (n=2 per vector).

Splenocytes and lymphocytes isolated from the draining lymph nodes of the injected limbs were combined for *in vitro* expansion of effector cells derived from each cohort of mice. Lymphocytes were plated out in a 24-well plate at a density of $5x10^6$ cells/well and cultured in 10% CO_2 at 37°C for 5 days. The cells were stimulated with hFIX secreted from C57SV (fibroblast) feeder cells infected with a retroviral vector expressing hFIX (MFG-hFIX). C57SV cells were seeded at a ratio of 1:50 with the lymphocytes. (Initially it was decided to use a feeder cell transfected with a plasmid-encoding factor IX, but this procedure failed to work despite repeated attempts. Therefore, a retrovirally-infected feeder cell was used instead). Prior to seeding, feeder cells were irradiated at 2000 Gy for 20 minutes to stop their proliferation *in vitro* during the culture period. Effector cells were harvested after 5 days, and a CTL assay was set up against H-2 compatible target cells (H-2b) infected with a retroviral vector expressing hFIX.

Generation of Target Cells

The target cell line TC-1 was transduced with a retroviral vector for expression of hFIX. Lung epithelial-derived TC-1 cells are derived from C57BL/6 mice and can serve as histocompatible target cells for $CD8^+$ effector T cells from this mouse strain (Lin *et al.*, 1996). Prior to seeding with the effector cells, target cells were checked for expression of FIX. Target cells (C57BL/6-derived lung epithelial cell line TC-1 (Lin *et al.*, 1996) were labelled with ^{51}Cr and subsequently incubated with effector cells at ratios of 100:1 to 0.75:1 (effector: target) for 5 hrs at 37°C in 10% CO_2. Specific target cell lysis was calculated as follows:

$$\left[\frac{\textbf{experimental release}_{\textbf{CPM}} - \textbf{spontaneous release}_{\textbf{CPM}}}{\textbf{maximum release}_{\textbf{CPM}} - \textbf{spontaneous release}_{\textbf{CPM}}} \right] X\,100$$

(**CPM** = counts per minute)

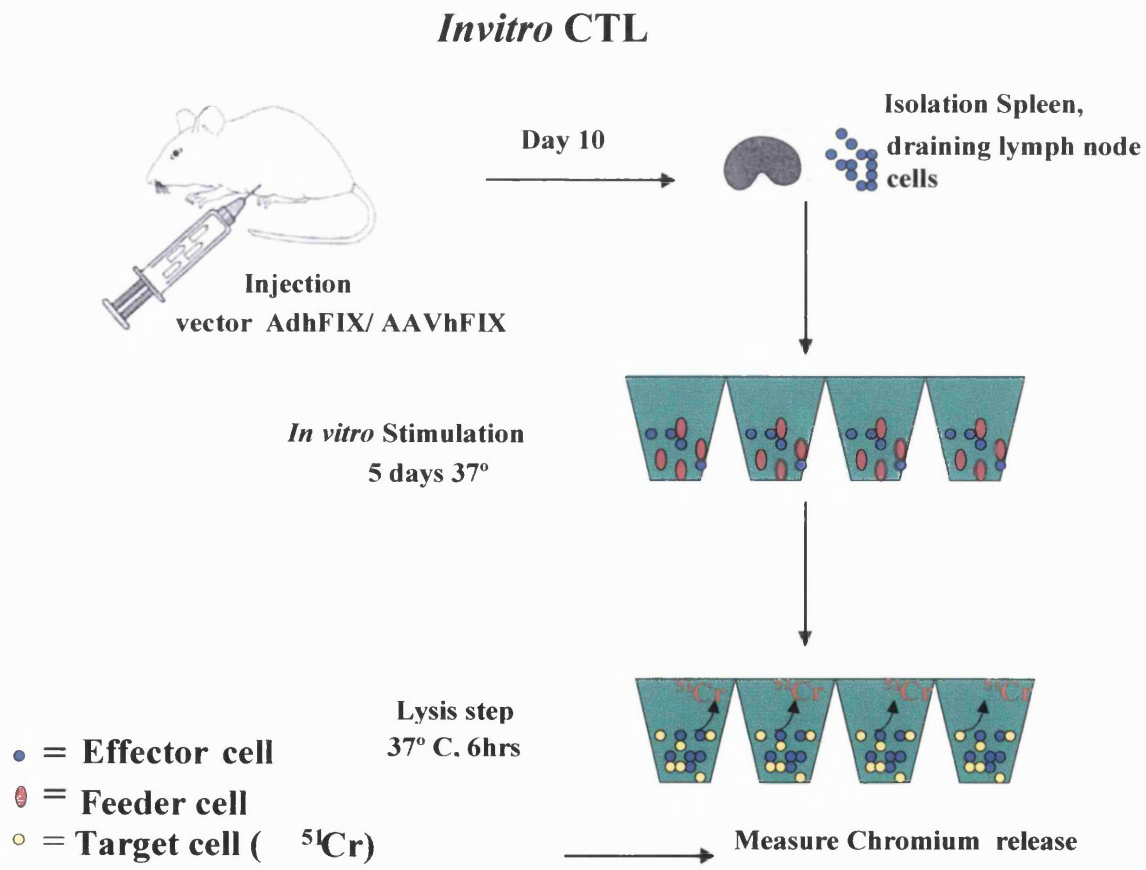
All experimental values given represented the average of three wells; maximum (i.e. target cells incubated with 10% SDS) and spontaneous (i.e. target cells incubated with medium alone) were also averaged from three wells. Spontaneous release was

consistently less than 30% of the maximum release. Standard deviation for triplicate measurements of ^{51}Cr release ranged from 1-16% of mean values.

Mock transduced target cells (i.e. target cells that are not expressing FIX) were used as a negative control for lysis by effector cells to account for non-antigen-specific lysis. Lysis of target cells by effector cells from uninjected mice was consistently ≤3%.

An outline of the experimental schema is shown below:

Fig 43 *In vitro CTL*



The invitro CTL is shown above. Briefly 2 cohorts of mive were injected with either an AAV or Ad vector encoding human factor IX. At day 10, the spleen and draining lymph node were removed and cell suspensions prepared to be used effector cells in an in vitro CTL assay.

Results

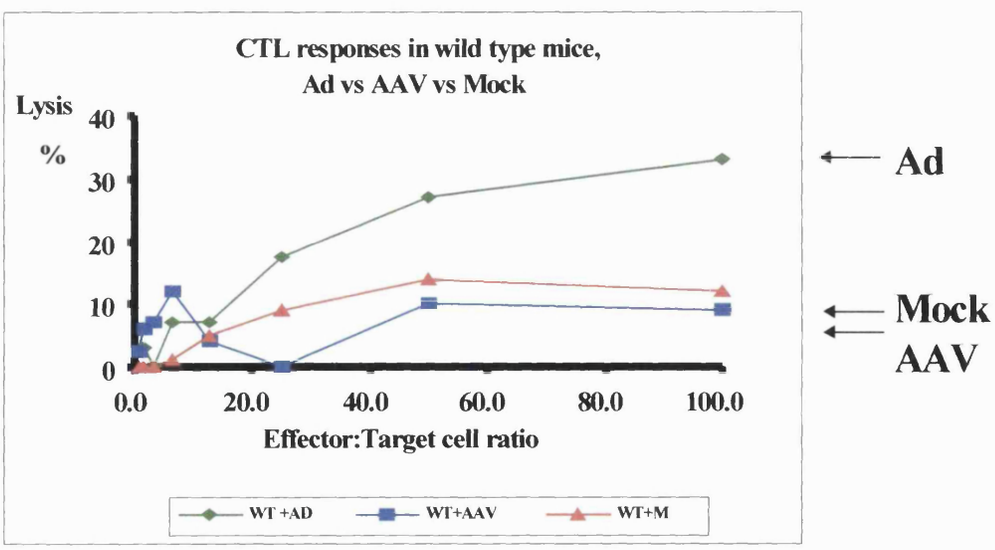
In vitro CTL

As judged by chromium release assay, adenovirus-injected normal and haemophilic mice developed hFIX-specific cytolytic activity causing lysis of up to 30-55% of target cells at the highest effector: target cell ratio (Figure 44a, b). Lysis of hFIX expressing target cells incubated with lymphocytes from AAV-injected normal or haemophiliac mice was similar to mock-transduced target cells indicating absence of FIX-specific CTL activity. Lysis of mock transduced cells is likely caused by NK cells, and is often found increased when effector cells from adenovirus immunised mice are used (HCJ Ertl, unpublished observations). These *in vitro* data correlated with infiltrates of $CD8^+$ T cells in adenovirus-injected muscle and absence of $CD8^+$ infiltrates in AAV-transduced muscle at the site of transgene expression (please see chapter 5,page 120).

Fig 44 *In vitro CTL results*

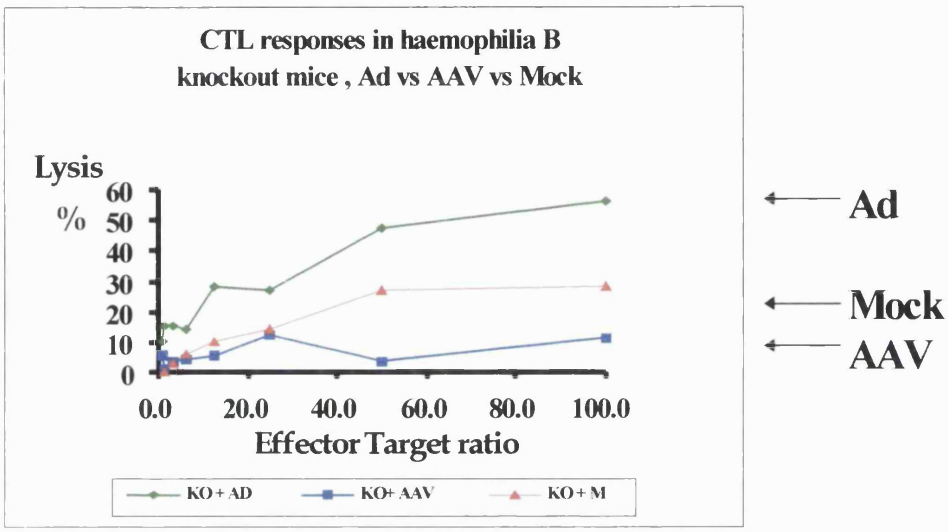
A)

Wild type C57BL6 mice: CTL responses in AAV, Ad immunised mice



B)

Haemophilia B knockout mice: CTL responses in AAV , Ad immunised



The graphs above show the percentage specific lysis of target cells as a function of effector cell: target cell ratio. ***A, B.*** *Lysis of hFIX-expressing TC-1 target cells (hFIX expression from a retroviral vector) after incubation with lymphocytes from AAV- (AAV-hFIX / FIX) or adenovirus -injected mice (Ad-hFIX / hFIX). Mice were normal C57BL/6 in graph* ***A*** *and hemophilia B mice on C57BL/6 genetic background in graph* ***B****. Mock controls represent lysis of untransduced TC-1 target cells (i.e. cells not expressing FIX) by lymphocytes from AAV- (AAV-hFIX / mock) or adenovirus - injected mice (Ad-hFIX / mock).*

Summary

As can be seen from the above graphs both wild type and haemophilia B animals immunised with the Adenoviral vector elicited strong CTL responses to the human factor IX transgene product. The AAV immunised animals however failed to elicit any comparable CTL response above negative control values. The data lend support to a relative absence of a CTL response via AAV mediated gene transfer. These results confirm histologically with a relative absence of inflammatory infiltrates seen in the AAV animals (Chapter 5, page 121).

6.3.1 Cytokine analysis of injected animals

As well as directly looking for a CTL response to the transgene product human factor IX, another way of dissecting out the immune response at a cellular level is to try and establish the cytokines expressed during the immune response by effector cell populations involved in the immune response. If a true CTL response was occurring CD4 T helper cells would secrete the Th1 cytokine IFNγ and IL-2, both known to correlate with CTL responses.

Method

Splenocytes and lymphocytes from the draining lymph nodes of injected muscle were cultured in DMEM (supplemented with 2% heat inactivated foetal calf serum and 10^{-6} M-2 mercaptoethanol) and restimulated *in vitro* with antigen. The antigens used were either Mononine (c2.5μg/ml) or heat inactivated AdhFIX vector (250 particles/cell). Cell free supernatants were harvested at 96 hours and analysed for the presence of IL-2, IFNγ, IL-4 and IL-10 by ELISA. Antibodies against murine cytokines and cytokine standards were provided by Pharmingen. Mock-stimulated cells were used as negative controls (for method seeChapter3, page 67-69).

Results

The results are shown in tabulated form below:

Table 7 *Cytokine release assay*

Stimulating Antigen :		FIX			Ad-FIX		
Injected mice	**VectorVector**	**IL-2 (pg/ml)**	**IFN γ (pg/ml)**	**IL-10 (pg/ml)**	**IL-2 (pg/ml)**	**IFN-g (pg/ml)**	**IL-10 (pg/ml)**
Wild type	**Ad**	**24**	**440**	**45**	**124**	**3200**	**65**
	AAV	**0**	**0**	**12**	**0**	**0**	**0**
HB mice	**Ad**	**22**	**260**	**17**	**39**	**996**	**163**
	AAV	**0**	**0**	**20**	**0**	**0**	**0**

As can be seen from the above table, the AdhFIX injected mice displayed high levels of the cytokines IL-2 and IFNγ in keeping with a Th1 (CTL) profile. In contrast the AAV injected animals did not elicit this type of profile and instead elicited a small amount of IL-10 expression more in keeping with Th2 response. These data support the CTL responses and histological findings (Chapter 5) observed for the Adenoviral injected animals.

6.4 *In Vivo* CTL

A possible confounding factor in the above results was that the above experiment represents an *in vitro* assay and may not be fully representative of the *in vivo* situation. Because of this, an *in vivo* CTL was set up to analyse whether the *in vitro* results could be confirmed.

The experiment performed was an adoptive transfer experiment, whereby lymphocytes from immunised animals were adoptively transferred into double knockout animals (Haemophilia/ Rag-1 double knockout) expressing high levels of factor IX to see if the recipient animal's factor IX levels could decreased by the adoptive transfer of the immunised animals lymphocytes. Haemophilia B/Rag-1 mice were generated by cross breeding of haemophilia B (HB) mice with immunodeficient Rag-1 mice. These mice lack mature B and T cells and have no circulating immunoglobulins (Mombaerts *et al.*, 1992). HB/Rag-1 mice were intravenously injected with the adenoviral vector resulting in high systemic expression of circulating hFIX antigen.

Pooled lymphocytes from AAV and adenovirus injected C57BL/6 mice (isolated on day 10 after IM vector administration, a time point when the mice do not have detectable anti-hFIX, n=3 for each set of mice) were adoptively transferred to the HB/Rag-1 double knockout mice (n=2 for each pool of lymphocytes) on day 1 of the experiment. Prior to transfer, splenocytes/lymphocytes were depleted for B cells using a CD45R antibody column. B cell depletion was confirmed by flow cytometric analysis The reason for B cell depleting the adoptivley transferred cells was to ensure that any oberved fall in circulating factor IX in the recipient animals was due to a

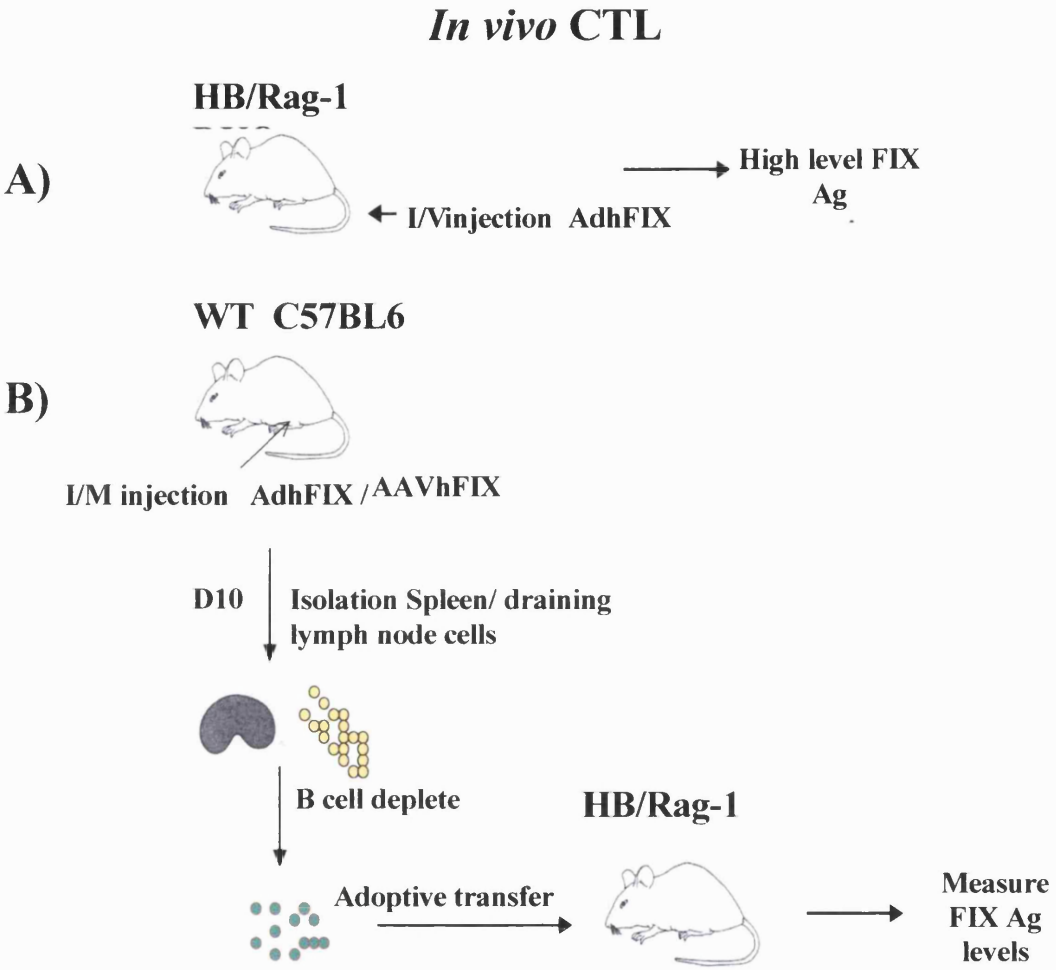
cellular response, and not a humoral response. To interpret the results of these experiments, if a true CTL had occurred in the immunised animals then the factor IX level would fall in the recipient double knockout animals. The experiment is described in more detail below.

Adoptive transfer of T cells

Haemophilia B/Rag-1 mice received intravenous injection of $1x10^{11}$ particles of ad-hFIX vector resulting in systemic expression of hFIX as confirmed by ELISA for hFIX in plasma samples. C57BL/6 mice received IM injections of AAV or adenovirus vector as described above (n=3 per vector) and were sacrificed at day 10 for adoptive transfer of B cell-depleted splenocytes/lymphocytes to hFIX expressing HB/Rag-1 mice. Draining lymph nodes (inguinal and popliteal nodes) of injected muscle were carefully dissected. Lymph nodes and spleen were isolated, crushed, and washed twice in lymphocyte separation medium (Amersham Pharmacia Biotech, Arlington Heights, IL). Isolated cells were pooled for each of the two cohorts of mice and run over a Ficoll gradient (Histopaque, Sigma, St. Louis, MO) to separate out red blood cells. The gradient was spun at 2000 rpm for 20 min, and the mononuclear cell layer was carefully aspirated off and washed twice. The isolated mononuclear fraction was incubated with micro beads coated with CD45R monoclonal antibody (clone B220, Miltenyl Biotech, Germany) at room temperature in order to remove B cells. B cell depletion was confirmed by flow cytometry using conjugated anti-CD3 and CD45R antibodies. Residual B cells were 2.9±1.1% of the final cell population.

Finally, a cell count was performed, and a total of $1x10^{6}$ cells were adoptively transferred into HB/Rag-1 via tail vein injection (100 µl cell suspension/mouse, n=2 for lymphocytes from each cohort of immunised C57BL/6 mice). Mice were subsequently bled from the tail vein one and four days after the procedure. A schematic diagram of the experimental procedure is shown below:

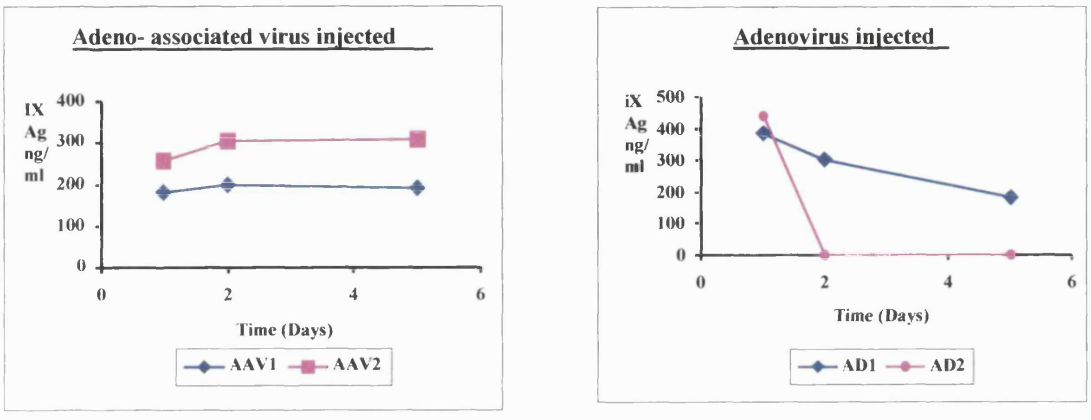
Fig 45 *In vivo CTL*



The diagram shows that the injected cohorts (wtC57BL6) spleen and draining lymph node cells were removed, and B cell depleted over a column. These T cells were subsequently adoptively transferred back into HB/Rag-1 double knockout animals, which were expressing high levels of circulating hFIX. Subsequently these FIX levels were measured in these animals to look to see if the FIX levels were decreased by the introduction of the adoptivley transferred lymphocytes i.e. whether a CTL was induced.

Fig 46 *In vivo CTL*

In vivo CTL: FIX levels post adoptive transfer



The results of the adoptive transfer shown above illustrate that in the right hand panel the adenoviral immunised animals dropped the factor IX levels in the recipient animals by 100% and 50% respectively. Conversely in the left hand panel it can be seen that the AAV immunised animals failed to decrease the factor IX levels in the double knockout animals.

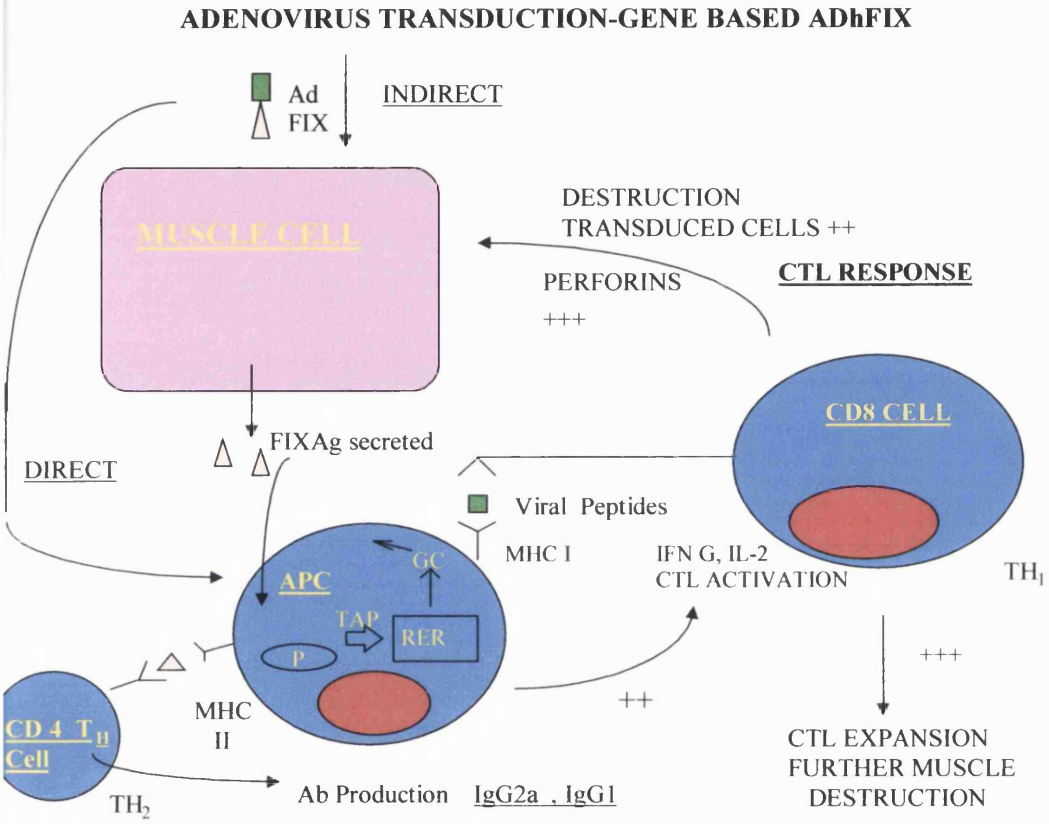
These results from the adoptive transfer experiment confirm that there appears to be no *in vivo* CTL response with the AAV viral vectors.

6.5 Discussion

These results presented in this chapter show the antigen (FIX) specific T cells adoptively transferred from the Adenoviral immunised animals elicited an *in vivo* CTL response in the double knockout animals, whilst the AAV immunised animals failed to elicit such a response. These results subsequently confirmed the earlier described *in vitro* findings. In summary therefore it appears that Adenoviral based muscle transduction induced a cellular Cytotoxic T cell response to the human factor

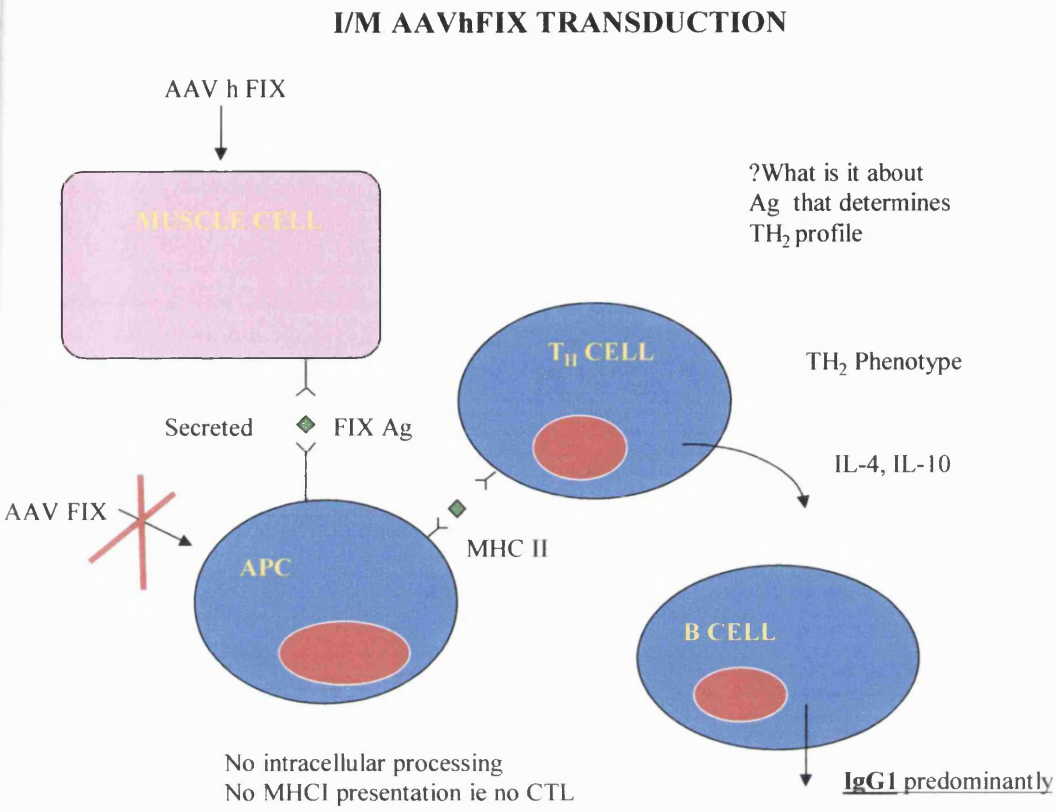
IX antigen whilst AAV based muscle transduction did not. The proposed mechanisms of both AAV and Adenoviral based muscle transduction are shown below:

Fig 47 *Adenoviral Transduced Muscle*



In the above diagram it can be seen that adenoviral based transduction is able to elicit both an MHC class I response resulting in the destruction of transduced muscle and also a class II response. In the above response two profiles of an antibody are produced (IgG1 and IgG2) in keeping with both a Th1 and Th2 response, as well as CTLs through MHC class I and II presentation of FIX expressed in APCs or secreted from transduced muscle cells. Immune responses against the transgene product are also likely critical for the elimination of transduced muscle fibres (Tripathy *et al.*, 1996). The CTL response is directed both to the viral proteins as well factor IX. Why a CTL response is induced here to the factor IX maybe because the Adenoviral based response may be sufficient to trigger a CTL response to the factor IX transgene by creating a sufficient cytokine milieu to induce this response.

Fig 48 *Adeno-associated based Transduced muscle*



In the above immune response it can be seen that a CTL response is not induced in response to AAV based transduction. The response induced is predominantly a TH2 driven cellular immune response as evidence by the humoral profiles demonstrated (IgG1). This appears to be the major difference from adenoviral based transduction. The mechanism as why this maybe the case is currently under study although a previous report documented that AAV vectors were relatively inefficient at transducing antigen presenting cells and so enter into an MHC class I pathway.

In the context of AAV vectors, only MHC class II presentation is part of the immune response, resulting primarily in activation of Th2 cells through uptake of muscle cell-derived FIX.

This model would predict that antibody production against a secreted protein is independent of protein synthesis in APCs and can therefore not be prevented by use of

a tissue-specific promoter (i.e. a promoter that is not active in APCs) as has been proposed for some gene therapy protocols (Pastore *et al.*, 1999). Additionally other factors such as immunogenicity of capsid proteins (Dai *et al.*, 1995), infection of cell types other than muscle fibres such as dendritic cells, and adjuvant effect from compounds present in the vector preparation may all play a role in immune responses to viral vectors.

Explanation for lack of cellular immunity to AAV based transduction

The lack of immune responses to the transgene product in AAV based muscle transduction is different to that observed in Adenoviral based transduction where transduction of APCs is required for T cell activation to the transgene

The mechanism of why AAV vectors at least in murine species fail to elicit activation of an MHC class I pathway is unclear, but it s been suggested that AAV fail to transduce APC cells efficiently (due to post entry block) and thus do not enter into a cytotoxic pathway.

One could therefore hypothesise that efficient transduction of APCs is defective in the context of AAV. This hypothesis was tested by Jooss et al (Jooss *et al.*, 1998c) in the following experiment. Naive APCs purified from spleen were exposed to high titre AAV prior to adoptive transfer into C57BL6 mice previously injected intramuscularly with AAV lac z. Direct analysis of the APCs failed to reveal evidence of B galactosidase expression. Furthermore these animals failed to activate T cells to ß galactosidase and expression of the transgene was stable.

Mechanism of defective antigen presentation of APCs

There are several mechanisms by which antigen presentation may be avoided in the context of AAV based gene transfer.

1) Insufficient activation of the APC for presentation of antigen

2) Suppression of antigen presentation

3) Inability of AAV to transduce APC.

The most likely reason for the observed difference in antigen presentation between Ad and AAV transduction is selective transduction of APCs by the different vectors. Transduction of fractionated populations of APCs with Adlacz demonstrated *lac* Z expression in dendritic cells and some macrophages, which effectively activated T cells to ß galactosidase following adoptive transfer. *Lac* Z expression was not detectable in APCs following exposure to AAVlacZ, nor were these cells capable of activating T cells *in vivo*.

These findings were essentially consistent with the notion that AAV efficiently enters a number of cell types, most of which are not permissive for transduction due to post entry blocks. The post entry bock was studied *in vitro,* which showed on FISH analysis that the vector genome localises in a perinuclear distribution. In order to be transcriptionally active the genome needs to enter the nucleus. Coinfection with an E1 and E4 expressing adeonovirus mobilised the AAV genome into intranuclear replication centres, which were transcriptionally active. The data produced from these experiments indicated AAV enters APCs in a non-productive transduction (Jooss *et al.*, 1998c).

The significance of the findings in this chapter point out the critical importance of vector selection and the influence this has on the cellular responses observed to gene transfer (Fields *et al.*, 2000). In particular and crucially for AAV vectors, there appears to be no CTL response elicited when these vectors are employed, so facilitating the long term gene expression observed with these vectors. These findings have wide implications for future human clinical trials.

CHAPTER 7

INHIBITOR FORMATION FOLLOWING GENE TRANSFER: CHARACTERISATION AND IMMUNOMODULATORY STRATEGIES

7.1 Background

The most devastating effect of replacement therapy in haemophilia is the occurrence of inhibitors, which occurs in up to 25% of patients with haemophilia A, and 3-4 % of haemophilia B. The presence of inhibitory antibodies to either factor VIII or FIX dramatically complicates therapy for these patients, with a consequent decrease in quality of life and life expectancy. The range of treatments available to treat inhibitor formation include the use of bypass therapy with activated and non activated prothrombin complex concentrates, high dose factor VIII, porcine factor VIII, and recombinant factor VII (Chapter 1, pages 28-32)

Several strategies have been used (so called "immune tolerance regimens") that aim to induce tolerance to exogenous clotting factor VIII or IX, and have included the use of very high doses of clotting factor concentrates alone, or in conjunction with immuno modulating agents. These treatment modalities are extremely expensive, require daily or twice daily administration and are not infrequently unsuccessful, especially when the inhibitor is well established and present in high titre.

Any novel treatment plan for haemophilia always raises concerns about potential inhibitor development, and therefore, at the outset of therapy the ability to predict which patients are at risk of inhibitor formation, and identify factors that predispose to inhibitor development would allow for the development of therapies which could either prevent or overcome this complication. The treatment of haemophilia by a gene-based therapy represents a new treatment strategy in which the risk of inhibitor formation is currently unknown. The work presented in this chapter is divided into 4 subsections.

- Present understanding of the development of inhibitors in conventional replacement therapy particularly in relation to haemophilia B
- The immunological pathways involved in inhibitor formation
- Possible strategies to immunomodulate inhibitor formation
- Experimental data
    a) Inhibitor formation in Haemophilia B mice without immunomodulation
    b) Inhibitor formation in Haemophilia B mice with immunomodulation

7.2 Aetiology of Inhibitor Formation

The aetiology of inhibitor formation is a complex issue. However several studies have tried to address the risk factors involved, which may be broadly divided into genetic and non-genetic causes.

7.2.1 Non Genetic factors

Several non-genetic factors have been studied, but none to date have shown clear associations with the incidence of inhibitor development. In general it is accepted that the type and purity of coagulation factor concentrates, age at the time of initial treatment, initial doses of concentrate, and frequency of dosing prior to inhibitor development have not been shown to be associated with risk (Hoyer, 1995).

Exceptions however, include cases whereby processing in manufacture lead to neo antigenicity of the clotting factor concentrate and subsequent inhibitor development. Two small clusters of patients, who had been previously tolerant to factor VIII infusion have developed inhibitors following infusion with new pasteurised factor VIII concentrates, which appeared to cause changes in the molecular structure leading to increased immunogenicity (Peerlinck *et al.*, 1993).

Other factors, which alter the immune characteristics of a susceptible individual, may lead to the generation of sufficient inflammatory signals and trigger off an immune response resulting in an inhibitor formation. Although the HIV epidemic in the early 1980`s did not affect the propensity to develop inhibitors it was later subsequently shown that the chronic HIV infections were associated with spontaneous disappearance of inhibitors in both high and low responder populations. The subsequent loss of CD4 positive T helper cells lends support to the notion that T cells are integral in the pathogenesis of inhibitor development (*vide infra*)

7.2.2 Genetic causes

In general the more severe the genetic defect, the greater the loss of coding information and the more likely the development of an inhibitor. Therefore it is

predictable that the risk of inhibitor formation is related to the underlying mutation .The incidence of inhibitor formation is much higher in haemophilia A (20%) (de Biasi *et al.*, 1994) than haemophilia B (4%) (Briet *et al.*, 1984) and this is, in the main is accounted for by the mutation type leading to the disease. The following table shows the prevalence of inhibitor formation in Haemophilia A and B and the frequency of genetic defects leading to severe disease.

Figure 49 *Mutation incidence in Haemophilia A and Haemophilia B*

	Haemophilia A	**Haemophilia B**
Prevalence of inhibitor formation	**20-30%**	**3-4%**
Frequency of large gene deletion/ inversion in severe disease	**40-50%**	**4-7%**
	Inhibitors in patients with •Large gene deletion:36% •inversion :35% •Stop mutation:38% (missense mutation:4%)	50-60% of inhibitor patients with hemophilia B have a large gene deletion

Nature of the underlying mutations in Haemophilia B

Based in the UK, the haemophilia B database published annually since 1990 (Giannelli *et al.*, 1994; Giannelli *et al.*, 1996; Giannelli *et al.*, 1997; Giannelli *et al.*, 1998), is now the largest database of disease-associated mutations, with 1713 patient entries representing 652 unique molecular events (Giannelli *et al.*, 1997). In a study performed by Sommer (Sommer 1992), 260 consecutive haemophilia B cases were sequenced (in terms of the predicted protein). 63% of the patients had mis-sense proteins, 30% garbled proteins (truncated, frame shift, or partial or full deletions), 7% abnormal splicing, and 0.6% decreased expression.

Mutation and Inhibitor risk in Haemophilia B

In terms of mutations associated with inhibitor formation, it was recognised early on that the presence of a large gene deletion constituted a risk; in the study of Green et al, 17/25 patients (68%) with a deletion involving at least one exon developed an inhibitor (compare to overall risk of 2-3%, *vide supra*) (Green *et al.*, 1991). Overall, deletions account for 5% of all mutations resulting in haemophilia B, but 50% of mutations in patients with inhibitors (Sommer and Ketterling 1996). In addition to large deletions, mutations associated with loss of coding information (frame shift and termination mutations) also carry an increased risk of inhibitor formation.

In a Swedish study (Green *et al.*, 1991), the *a priori* risk of inhibitor formation in a patient with an unknown mutation was 3% but near zero for a patient with a mis-sense mutation and ~20% for those with mutations resulting in loss of coding information. Similar results were observed in a study of 18 children who exhibited anaphylactic reactions to FIX on developing an inhibitor (Warrier, 1998; Warrier & Lusher, 1998). 10/18 of the children carried a large gene deletion.

Although 50% of inhibitors in haemophilia B are accounted for by large gene deletions the rest are largely due to point mutations. These are listed in the haemophilia B database (Giannelli *et al.*, 1997) and some are described in table 8. It should be noted the database specifically excludes large gene deletions. It can be seen that inhibitor formation is largely confined to cases where there is an absence of detectable antigen. The single exception to this occurs in a mutation that alters the active site serine, an invariantly conserved residue in all the serine proteases.

Table 8 *Point mutations in the human FIX gene associated with inhibitors*

Patient	Act	Ag	Nucleotide Position & Mutation	Amino Acid Change	Comments
UK 12	<1		6,392, Δ 1	6	Frame shift
Madrid 3	<1	<1	6,401-10,Δ10	9	Frame shift
Bonn 2	<1	<1	6,402-6, Δ5	9	Frame shift *de novo* in mother
Chongqin	<1	<1	6,460,C →T	29,R→Stop	16 cases including 2 inhibitors
HB7, Japan	<1	<1	6,680-1, Δ 2	39	Frame shift terminates at aa 46 >100 Bethesda units
HB5, Japan	<1	<1	20,551, C →T	191,Q→S	>100 Bethesda units
unnamed	<1	<1	20,551, C →A	191,Q→K	
Malmö 5	<1	<0.1	20,561, G →A	194,Stop	
HB6, Japan	<1	<1	30,821, G →A	Destroys AG splice site	>100 Bethesda units
UK 140	<1	<1	30,863, C →T	248,R,Stop	15 cases including 1 inhibitor
Malmö 1	<1	<0.1	30,950-7,Δ8	277	Frame shift
Varel 1	<1	86	31,213-14, TA→CG	365,S →G	Active site, silent mutation at aa364;

Mutation incidences in Haemophilia A

Schwaab (Brackmann *et al.*, 1993) and colleagues have reported that intra chromosomal mutations were associated with inhibitor formation in 42.4% of patients, whilst stop mutations and large gene deletions with 35% and 23.1% incidence respectively, whereas mis-sense mutations and small gene deletions were associated with only 6.7% and 9.1% incidence of inhibitors respectively. The currently accepted hypothesis is that large gene deletions, gene inversions and stop mutations lead to no circulating factor VIII protein and therefore substituted FVIII factor represents a foreign protein evoking a host immune response.

In contrast mis-sense mutations possibly result in circulating non-functional factor VIII protein creating immune tolerance in most of these patients. Despite the clear association with underlying mutation status, it still remains unclear why only 42% of patient and not 100% of patients with a large gene inversion develop inhibitors.

7.3 Other Inherited Characteristics

7.3.1 HLA Association

Several investigators have examined the possible role of the MHC locus in inhibitor formation; particularly MHC class II molecules since they are involved exogenous antigen processing. However no correlation of DR types in inhibitor versus non-inhibitor patients has been found. However, the earlier studies were not able to take into account the mutation type. It is possible therefore diverse molecular defects may have interfered with the ability to closely examine the influence of immune response genes in inhibitor formation.

To address this problem, the influence of HLA genotype(Oldenburg *et al.*, 1997) was investigated exclusively in patients with an intron 22 inversion as a uniform mutation type. Seventy-one patients were studied, 42 without and 29 with inhibitors (13 high, 9 low, and 7 transient responders) and were genotyped for MHC Class I HLA –A, B, C and class II HLA –DQA, DQB, and DRB loci. No strong correlation of any HLA allele to inhibitor or non-inhibitor status was demonstrated. However alleles of the haplotype HLA-A3, HLA-B7, HLA-C7, HLA –DQA0102, HLA-DQB0602, HLA-DR15 were more often found in inhibitor patients. These alleles are more common in those of Northern European descent suggesting a racial predisposition.

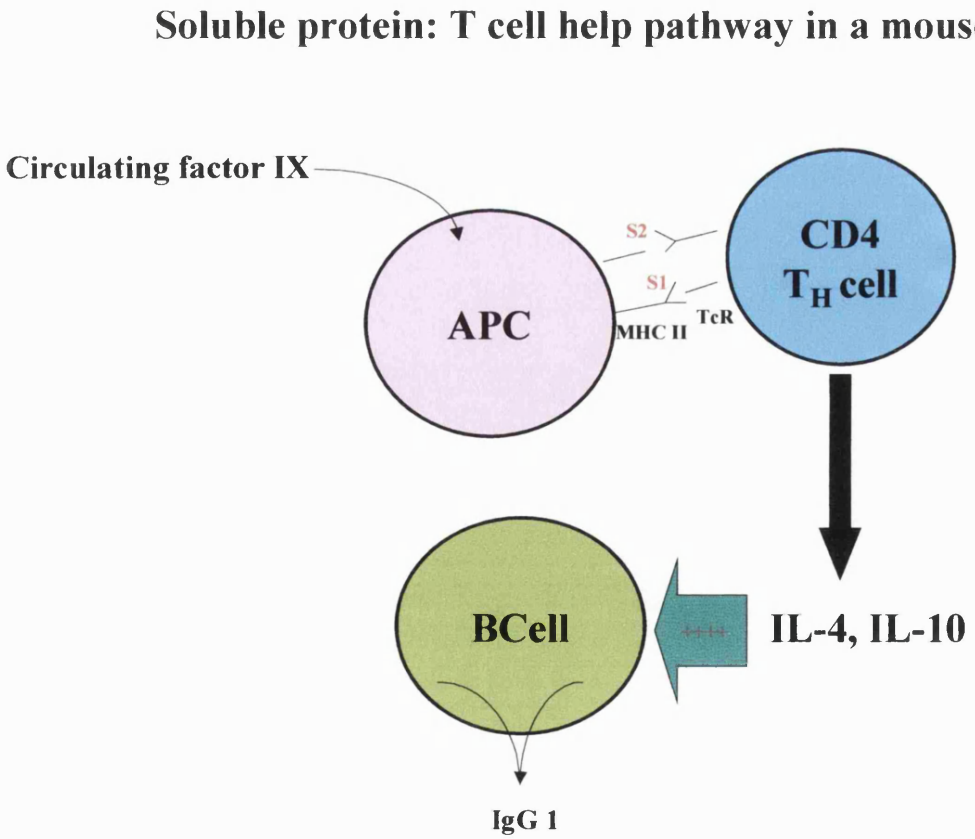
In a further study, demonstrating the importance of race, a Japanese group (Ohta et al) serologically (HLA, A, B, Cw, DR and DQ) tested and genotyped (HLA –DQA1, DQB1, DRBV1 and DPB1) 46 patients. 20 of these patients developed an inhibitor to FVIII, but unlike Oldenburg`s study the genotype was not constant and 9/20 patients had a gene deletion, 6/20 a gene inversion, and no deletion or inversion in 4 patients and unknown status in one patient.

In this study, it appeared that absence of the HLA-A24 allele was a principle risk factor for inhibitor development (p=. 0003). Other haplotypes HLA-DR4.1, DQ4, and DQA1*0301=2 were positively associated with patients developing inhibitors when compared to normal subjects. The authors concluded that the association between HLA antigens and the formation of inhibitor was dependent on racial background.

7.4 Pathways Involved in the Immune Response

In the conventional approach to treating haemophilia, the immune response represents the response observed to a soluble protein i.e. infused clotting factor. In this response there several different stages of the process involved in immune recognition and an immune response. There are likely to several different cell populations involved before the generation of an antibody response. Clotting factor protein antigens are taken up by an antigen-presenting cell and then presented in the context of MHC class II molecules to effector CD 4+ cells. The simplified process is shown schematically below:

Figure 50 *CD4 T cell help pathway*



As can be seen from the above diagram, there are three cell types central to the above process a) APCs, b) CD4+ cells, and c) B cells and subsequent antibody production. Characterisation of these cellular interactions will provide clues to the aetiology of inhibitor formation, and offer ways of developing therapies to overcome the response. Each of these components will now be considered in more detail.

7.4.1 Antigen processing pathways

Before the effector immune system actually meets antigen, several things occur to the antigen before it is ready to be presented to this system. Antigen processing is highly complex and ordered process. T lymphocytes recognise peptide fragments that are bound to cell surface proteins encoded by genes of the major histocompatibility complex (MHC). MHC molecules serve to display peptides to T cells. Peptides derived from cytosolic proteins are bound to MHC class I molecules and are recognised by CD8+ T cells, which are usually cytolytic T cells (CTLs). CTL` s provide the major host defence system against intracellular microbes which produce proteins in the host cell cytoplasm. Therefore proteins made by the intracellular introduction of transfected genes via a gene transfer process, may enter the pathways described above, and generate a cellular cytotoxic T cell response (CTLs).

In contrast, peptides derived from extracellular proteins such as infused clotting factor proteins are endocytosed by specialised cell types and bound to class II molecules and are recognised by CD4 +T cells, which are usually helper T lymphocytes. Helper T cells are most effective in eliminating extracellular and phagocytosed antigens.

Antigen Presenting cells

The generation of complexes of MHC molecules and peptide antigen on the surface of APC`s involves intricate multistep pathways within the APC. In general, both MHC I and MHC II pathways have features in common:

1) Peptide antigens are generated by proteolysis of intact proteins within subcellular organelles of APCs

2) Peptide binding to MHC molecules occurs prior to cell surface expression.

3) Both class I and class II MHC pathways of antigen processing and presentation utilise subcellular organelles and enzymes that have generalised protein degradation and recycling functions that are not exclusively used for antigen display to the immune system.

4) Both class I and class II antigen processing and presentation pathways do not distinguish self proteins from foreign antigens but rather display peptides from a sampling of all endocytosed and cytoplasmic proteins for T cell surveillance.

Types of APC

The two requisite properties that allow a cell to function as an APC for class II MHC restricted helper T lymphocytes are the ability to process endocytosed antigens and the expression of class II MHC gene products. The best-defined APCs for helper T lymphocytes include three cell types classified as *Professional* antigen presenting cells: Dendritic cells, macrophages, and B-lymphocytes.

The distinguishing feature of these cells is their ability to express class II MHC molecules and their ability to deliver a co stimulatory signal (*vide infra*). These cells differ in their mechanisms of antigen uptake, in whether they constitutively express class II MHC molecules, and in their co stimulatory activity: Dendritic cells are the most effective antigen presenting cells, and because they constitutively express high levels of class II and co stimulatory activity, they can activate naïve Th cells. Macrophages must be activated by phagocytosing microorganisms before then expressing class II MHC molecules or the co stimulatory B7 membrane molecule. B cells constitutively express class II MHC molecules but must be activated before they express the co stimulatory B7 molecule. Several other cell types, classified as non-professional antigen cells, can be induced to express class II MHC molecules or a costimulatory signal.

Antigen presenting cells

Professional antigen presenting cells	***Non professional*** antigen cells
Dendritic cells	Fibroblasts
Macrophages	Glial cells
B dells	Pancreatic beta cells
	Thymic epithelial cells
	Thyroid epithelial cells
	Vascular endothelial cells

Therefore in the consideration of an inhibitory immune response, it is important to consider where the possible response is taking place, and what kind of antigen presenting cells are accessible i.e. whether they are professional or non professional. In a gene-based approach the target tissue selected for gene expression will differ in its cellular APC populations, and the capacity to mount an effective immune response may not or may be present.

7.4.2 CD4 T+ helper cell activation

Antibody responses to protein antigens are considered T cell dependent since they require the participation of antigen specific T helper cells (van den Eertwegh *et al.*, 1992; Romagnani, 1996; Romagnani, 1997). These T cells provide signals essential for B cell activation and subsequent differentiation towards antibody secretion. Once engaged with an APC, CD4+ T helper cells are activated to produce cytokines and to proliferate in response to recognition of specific antigen, in the form of processed peptides bound to class II MHC proteins on antigen presenting cells (B cells, dendritic cells and macrophages).

Individual murine CD4 + cells can be broadly grouped into two major classes based on the production of certain representative cytokines in a mutually exclusive fashion. Th1 cells which produce IL-2 and IFN gamma and Th2 cells which produce IL-10 and IL-4(Romagnani, 1996; Romagnani, 1997; Romagnani *et al.*, 1997). Th0 have the ability to produce both sets of cytokines.

An individual T cell has the ability to acquire either a Th1 or Th2 phenotype. If T cell activation occurs in an environment rich in IL-12 and IFN γ, development of the Th1 phenotype is favoured whereas in the presence of IL-4 the Th2 phenotype is favoured.

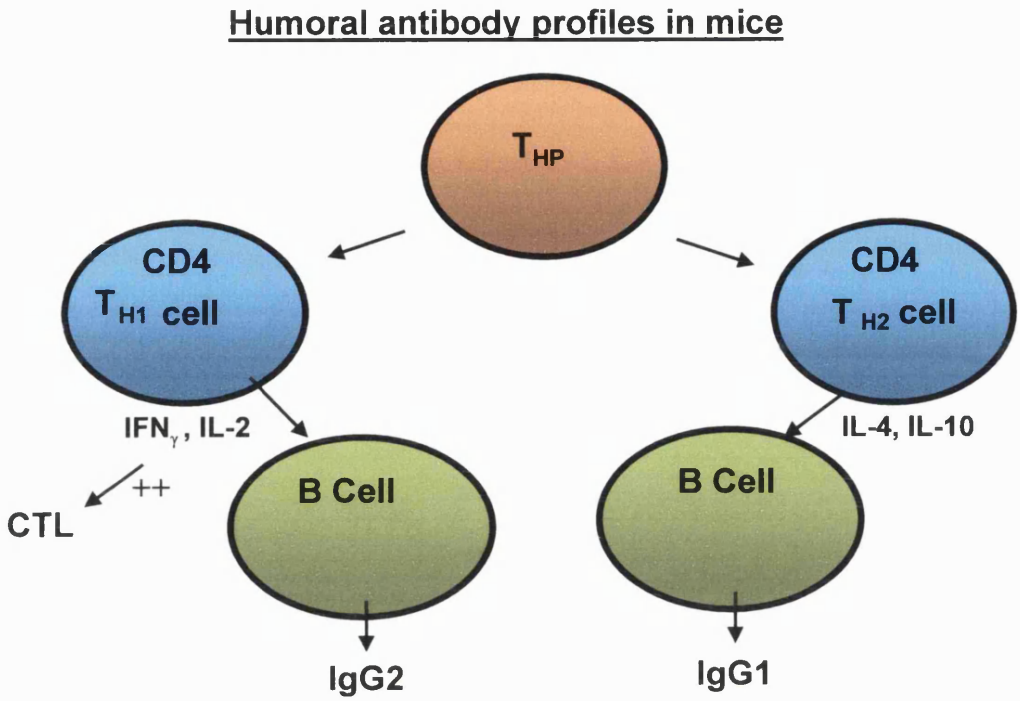
7.4.3 Role of Antibody Subtype and the immune response

As stated above, the immune response to soluble protein antigens is a T cell dependent antibody driven cellular humoral immune response. The nature of the underlying antibody subclass induced can suggest which type of T cellular subsets humoral response are involved in antibody generation. In man there are four antibody subclasses IgM, IgD, IgG1, IgG2, IgG3, IgG4, IgE, IgA1 and IgA2;

With rare exceptions the inhibitory antibodies in man are found to be of the IgG subtype and are predominantly of the IgG4 subclass. This finding suggests that that induction of the humoral response is T cell dependent and characteristic of a Th2 cellular immune response. i.e. it requires the presence of IL-4, IL-10 to provide help for B cell differentiation, maturation and subsequent isotype switching. Additionally the fact that certain patients with inhibitors can be made tolerant to infused clotting factor concentrates also suggests that T cells play a role in the development of antibody production.

Mice have antibodies of the classes IgM, IgD, IgG1, IgG2b, IgG2a, IgE, IgA and IgA; In the mouse, T cell dependent humoral responses are dominated by IgG1, IgG2a, IgA, and IgE (Severinson *et al.*, 1982; Tesch *et al.*, 1984). By characterisation of the antibody subclass induced in an immune response, it is possible therefore to extrapolate and determine which CD4 T cell helper subsets involved in the pathogenesis of an inhibitory antibody response. The diagram below summarises the CD4 T cellular responses in mice and their correlation with antibody profiles. This information is useful to the later work presented in this chapter, since the information yielded by the antibody subtypes induced by different vector selection was used initially as clue to which type of immune response was occurring prior to the development of cellular assays (CTL) which could answer definitively whether a cellular response was occurring (Chapter 6, page 152-160).

Fig 51 *Humoral profiles in mice*

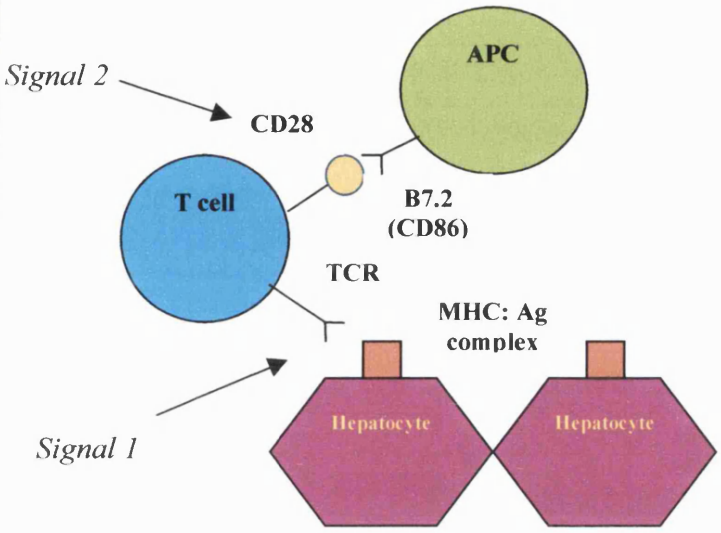


As can be seen from the above diagram the presence of an IgG1 response correlates with a Th2 cellular humoral response whilst an IgG1 response correlates with a Th1 driven cellular response. Therefore in a humoral (antibody response) occurring in mice, the antibody profile generated in this type of response would be an IgG1 profile. Conversely, a cell-mediated response (CTL) would correlate with an IgG2 (IgG2a/ IgG2b) profile.

7.4.4 The requirement for Costimulation in T Cell Activation

A general property of lymphocytes including both B and T cells is the need for two distinct extracelluar signals, in order to induce proliferation and differentiation into effector cells (Bretscher, 1992; Lafferty & Gill, 1993). The first signal is provided by antigen binding to the antigen receptor .In the case of T cells, peptide-MHC complex binding to the TCR (and CD4 and CD8 co receptors) provides *signal 1*. The second *signal 2* for T cell activation is provided by co stimulatory molecules, which are surface molecules on APCs, which bind to specific receptors on the T lymphocyte. A simplified diagram of this process is shown below:

Figure 52 *Costimulatory Pathways*



T cell activation requires two signals. ***Signal 1*** *is the presentation of a peptide fragment from the foreign protein, displayed in the context of an MHC molecule, to the T cell receptor (TCR).* ***Signal 2*** *is a co stimulatory signal from an antigen-presenting cell (APC)*

The importance of co stimulatory signals is evidences by what happens to T cells in their absence. By this approach two important roles for costimulation have been established:

1) Co stimulatory signals are required, concurrent with antigen induced signals in order for T cells to achieve full activation responses. When T cells recognise foreign antigen in the absence of costimulation i.e. they receive signal 1 without signal 2, they do not become fully activated to perform effector functions. This can be shown experimentally by treating pure populations of CD4 + cells with TCR agonist ligands such as polyvalent anti CD3 in the absence of any accessory cells. Under these conditions, the T cells produce very few cytokines and do not proliferate. If a source of co stimulatory signals, such as monocytes is added the T cells will respond vigorously to the anti CD3.

2) A lack of costimulation at the time of antigen presentation may eliminate a T cell from the pool of antigen responsive lymphocytes either by promoting its death or by inducing a state of unresponsiveness (*anergy*)

The expression of co stimulators is restricted to certain cell types and is highly regulated, Although there are various molecules which have been shown to have co stimulatory properties, the most potent co stimulators are expressed at high levels only on professional APCs such as mononuclear phagocytes, activated B cells and dendritic cells. Costimulator expression is often on resting APCs and is upregulated by stimuli such as cytokines, which accompany inflammation. These properties therefore profoundly regulate T cell mediated responses in the following ways:

a) The regulated expression of co stimulators ensures that T lymphocytes are activated at the correct time and place. An example being that a time of infection both microbial products and cytokines elaborated by inflammatory cells will upregulate costimulator expression on local APCs and thereby promote appropriate T cell activation to microbial antigens.

b) The coexpression of costimulators amplifies interactions between T cells and B cells and between T cells and macrophages. As well as serving as professional APC`s, both B cells and macrophages are recipients of T cell help i.e. they respond to products of activated T cells by themselves becoming activated and performing their functions. Therefore co stimulator expression on these APCs promotes activation of both the T cells and the APC`s.

c) The absence of co stimulators on unactivated or resting APCs in normal, uninflamed tissues contributes to the maintenance of tolerance to self-antigens.

Costimulatory signals for T cell activation are mediated by the molecular interactions between receptors on T dells and ligands on APCs. A key costimulatory signal results from the binding of CD 28 and cytotoxic T lymphocyte antigen (CTLA) –4 (CD152) receptors on T cells to CD80 and CD86 molecules are often referred to using trivial names, B7.1 and B7.2 respectively. One of the best characterised and most important

co stimulatory pathway in T cell activation involves the T cell surface molecule CD28 which binds the co stimulatory molecules B7.1 (CD80) and B7.2 (CD86) expressed on APCs. CD28 delivers signals that enhance T cell responses to antigen. During the process of an immune response either CD80 or CD86 molecules on APCs can provide costimulatory signals for T cell activation (Linsley, 1995a; Linsley & Goldstein, 1996). These are surface molecules on APCs, which bind to specific receptors on the T lymphocytes. CD80 and CD86 are members of the immunoglobulin superfamily that share limited sequence homology in the extracellular domains. These molecules are expressed on the surface of activated B cells, dendritic cells and macrophages, but exhibit distinctive patterns of expression and regulation. CD86 is reported to be constitutively expressed in some APCs such as dendritic cells and monocytes and rapidly upregulated upon activation. The kinetics of upregulation of CD80 is slower than of CD86.

In addition, another T cell surface molecule, CTLA- 4 also binds B7.1 and B7.2 but in contrast to CD28 it transmits signals that inhibits T cell activation. In addition to the effects of cytokines, the initial activation of CD4 + Th cell and subsequent responses by Th1 cells are markedly enhanced and in some cases dependent on concomitant engagement of CD28 on T cells by B7.1 or B7.2 molecules on antigen presenting cells. B7.2 engagement may favour the development of Th2 cells whereas later and more persistent B7.1 engagement may favour the development of Th1 response.

On mature dendritic cells the surface expression of CD86 is 10 times greater than that of CD80 and contributes to about 90% of CD28/CTLA –4 binding. Activated B cells macrophages and mature dendritic cells have relatively high surface CD80 and CD86 and are therefore strong activators of T cells. These cells are often referred to as professional antigen presenting cells. In contrast resting B cells and immature dendritic cells express MHC molecules but have low levels of CD80/CD86 on their cell surfaces. These cells present antigens poorly and probably induce T cell tolerance. These APCs are referred to as non-professional APCs. Costimulatory signals from antigen presenting cells have a central role in regulation of T cell tolerance in the periphery (Lenschow & Bluestone, 1993; Lenschow *et al.*, 1994). Thus, it can be seen that interference with these pathways may induce tolerance. (*vide infra, p187*).

7.4.5 Mechanism of tolerance and inhibitor development

One of the cardinal features of the immune system is its ability to recognise and respond to foreign antigens but not to self-antigens. This is called self- non self-discrimination. The unresponsiveness of the immune system to antigenic stimulation is called immunological tolerance. The phenomenon of self-discrimination is evident for a protein such as factor IX where this protein in normal individuals is non-immunogenic. However, in a FIX haemophiliac challenged with replacement therapy with factor IX, this may evoke an inhibitory antibody response. The challenge for any haemophilia therapy is to allow tolerance to the novel factor IX therapy, whether it is derived from a protein or gene based approach.

Immunological tolerance is the phenomena of antigen induced functional inactivation or death of specific lymphocytes resulting in the in ability of an organism to respond to that antigen. Lymphocyte activation and tolerance are two possible results of specific recognition of antigens by lymphocytes. Antigens that induce tolerance are called tolorogens, to be distinguished from immunogens, which generate immune responses. Tolerance to self-antigens is a fundamental property of the immune system, its failure resulting in autoimmune disease. Normally all self-antigens act as tolorogens. Many foreign antigens can be immunogens or tolorogens depending on their physiochemical characteristics, form, dose and route of administration.

The *general properties* of immunological tolerance are outlined as follows:

1) Tolerance is immunologically antigen specific and therefore must be due to the deletion or inactivation of antigen specific T and / or B-lymphocytes. Both lymphocyte activation and tolerance are induced by interaction of antigens with the same types of clonally distributed receptors on antigen specific cells i.e. membrane immunoglobulin on B cells, or the T cell receptor on MHC restricted T cells

2) Immature developing lymphocytes are more susceptible to tolerance induction than are mature or fully competent cells. During their normal maturation in the generative lymphoid organs, all lymphocytes go through a stage at which antigen

recognition lead to their death or inactivation. At this stage potentially self reactive lymphocyte clones encounter self- antigens and become tolerant to these antigens. This type of tolerance, which is induced by immature lymphocytes within the generative lymphoid organs, has been called ***central tolerance***. Central tolerance is important for maintaining unresponsiveness to self-antigens that are present at high concentrations in the generative lymphoid organs, but it does not play a role in tolerance to foreign antigens administered in the periphery.

3) Tolerance to foreign antigens is induced even in mature lymphocytes when these cells are exposed to antigens under particular conditions. Tolerance induced in mature lymphocytes that encounter antigens in peripheral tissues is called ***peripheral tolerance.***

Tolerance and inhibitor formation

From the standpoint of the immune response, inhibitor formation in haemophilia can be viewed as an appropriate response to a foreign protein, particularly in the case of patients with mutations that result in the loss of coding information. Tolerance to a protein is a complex phenomenon that is closely linked to antigen presentation (Matzinger, 1994; Matzinger, 1998) (Rammensee, 1996). For T cells, antigen presentation is dependent on the major histocompatibility proteins, which bind and display peptide fragments. Class I proteins, found on nearly all cells, bind peptides derived from proteins synthesised in the cell that displays them, while class II proteins display peptides from proteins taken up from the environment. Class II expression is normally confined to monocytes, dendritic cells, and B cells. Peptide bound to class I protein is recognised primarily by CD8+ T cells, while peptide bound to class II protein is recognised by CD4+ cells.

Tolerance, or unresponsiveness, can be due to one of several mechanisms. During foetal development, presentation of self-antigens in the primary lymphoid organs (the thymus for T cells, bone marrow for B cells) results in cell death (*clonal deletion*) or inactivation (*clonal anergy*) of those clones with high affinity for self. Other lymphocytes with lower affinity for self-antigens may persist, because the interaction falls below a threshold needed to trigger either deletion or anergy. This phenomenon, where cells have the potential for recognition of self, but fail to respond, has been

termed clonal "ignorance" (Nossal, 1993) under certain circumstances, *e. g.* a marked increase in antigen concentration, these lymphocytes may no longer "ignore" the stimulus. Finally tolerance may result from suppression, or immunoregulation, in which T cells actively suppress the immune response to antigen by a mechanism that is not well understood. Sustained production of antibodies on exposure to antigen requires promotion of B cell activation by helper T cells.

Recognition of an immunodominant epitope by T helper cells would result in T helper cell activation. For mutations that allow expression of even low levels of an immunodominant epitope, deletion of responsive T cell subsets can occur during foetal development. However, for mutations associated with complete absence of expression of these epitopes, one would predict a higher likelihood of inhibitor formation.

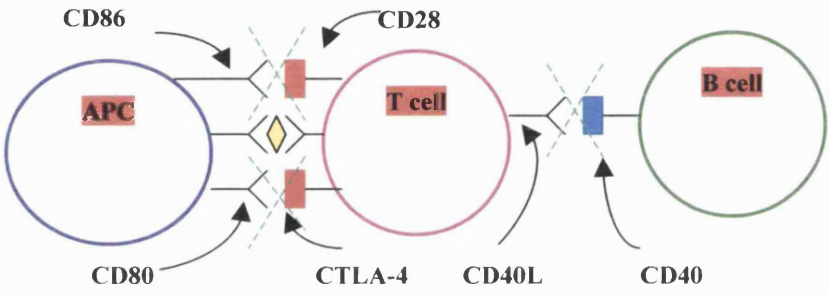
With respect to haemophilia, normally self-reactive T cells (i.e. such as those against clotting factors) are deleted or anergised during T cell development. However, individuals with haemophilia however because of their inherent genetic defects may not express the sequences i.e. the epitopes that are recognised by T cells specific to the wild type protein. Thus, these T cells mature in haemophiliac patients and on subsequent encounter with the antigen i.e. with replacement therapy promote induction of a neutralising antibody response.

Breaking of Tolerance

In order to avert inhibitor formation, it is necessary to induce tolerance to newly introduced clotting factor proteins whether derived from protein replacement therapy derived from a gene-based approach. There are numerous experimental systems for inducing tolerance to protein antigens. These include the administration of large doses of antigens without adjuvants, oral antigen administration, repeated exposure to the antigen and administration of mutated forms of the antigen. In all these situations tolorogens induce unresponsiveness by inhibiting the proliferation and differentiation of CD4 + T cells or by stimulating regulatory cells.

From the point of view of haemophilia, tolerance to infused protein antigens will require that T cells do not become activated and go onto developing an immune response. There are several points at which the failure of activation could take place. Simply blocking the signal one or signal two i.e. blocking the co stimulatory signal. These costimulatory signals are required for T cell activation. The diagram below illustrates the pathways of T-B cell interaction and the possible modes of interference in these pathways, which may lead to failure or of either T or B cell activation, and subsequent tolerance induction.

Figure 53 *Costimulatory pathway blocking*



It can be seen therefore, that in order to induce tolerance to clotting factor proteins, interference with co stimulatory pathways and the blocking of signal 2 (shown by dashed green lines in the figure above) may result in T cell anergy and the failure of B cell activation and subsequent antibody production.

As well interfering with T cell activation from CD80/CD86 pathways, as can be seen from the above diagram interference with CD40L pathway may lead to failure of B cell activation (Armitage *et al.*, 1992; Noelle *et al.*, 1992a; Durie *et al.*, 1994a; Durie *et al.*, 1994b). Cognate interaction of B cells with CD4 Th helper cells requires that the T cells express a protein ligand, CD40 ligand that engages CD40 on the surface of the B cell. Ligation of CD40 and interaction of antigen with surface immunoglobilin are sufficient to induce robust B cell proliferation to enhance the amount and affinity of antibody produced and to allow for B cell to switch production of antibodies from IgM to IgG, IgA or IgE production. Therefore, these bi-directional signals between T

and B cells (and other antigen presenting cells) are central to the efficient development of *de novo* cellular and humoral immunity to protein antigens.

7.5 Characterisation of Inhibitory Responses in A Gene Based Approach

In order to study the occurrence of inhibitor formation in a gene based approach, a series of experiments were carried out to characterise firstly the occurrence of inhibitor formation in both normal and haemophilia B mice models. The central hypothesis tested was to determine the incidence of antibody formation in a gene-based approach, and factors that affected it. In particular, the protein based approach has demonstrated that underlying mutation status correlates with likely inhibitor outcome with those mutations carrying a greater loss of coding material resulting in a more likely occurrence of inhibitor development.

7.5.1 Haemophilia B mice studies

From the normal mice studies, follow up studies were performed in Haemophilia B mice bred on a C57BL6 background. Haemophilia B mice were generated by gene targeting techniques in the laboratory of Dr Darrel Stafford (Lin *et al.*, 1997). These animals carry a large gene deletion effecting exons 1-3 of the factor IX gene which results in no FIX mRNA transcripts being made with the result that these animals produce no circulating factor IX antigen. They display a severe Haemophilia B phenotype, which mirrors the human condition. One would predict therefore that expression of circulating factor IX antigen via a gene-based approach in these animals would elicit antibody formation.

The experimental design of these experiments was to inject Haemophilia B mice with an AAV construct encoding murine factor IX and follow the animals out post injection to observe and characterise the occurrence of inhibitor formation in Haemophilia B mice carrying a large gene deletion for murine factor IX

Method

9 male Haemophilia B mice aged 8 weeks were injected intramuscularly at day 0 with an AAV vector encoding murine factor IX at a dose of $4x10^{10}$ vg/kg. The animals

were first anaesthetised with inhalational mefofane before injection. The genotype was determined by two sets of PCR reactions (one specific for the FIX wild-type allele, one specific for the knock out allele) on genomic DNA isolated from blood samples. Amplification primers and PCR conditions were as reported previously (Mombaerts *et al.*, 1992)

Vectors

A plasmid encoding AAV vector AAV-CMV-mFIX contains the murine FIX cDNA (2.7-Kb *Bam*HI fragment) under transcriptional control of the cytomegalovirus IE enhancer/promoter, and includes a chimeric CMV/β-globin mini-intron (5' to the mF IX cDNA), and the human growth hormone polyadenylation signal. The expression cassette is flanked by AAV serotype-2 inverted terminal repeats (ITRs). AAV-CMV-mFIX was produced by triple transfection of HEK-293 cells in a helper virus-free system (Matsuhita 1998, 1999), and purified by repeated CsCl gradient centrifugation (described in Chapter 4, Page 95-107). Vector titres were quantitated by slot blot hybridisation. Purified vector was stored at –80°C in PBS containing an osmotic stabiliser, and further diluted with sterile PBS prior to injection of mice.

Antibody determinations

The presence of antibody was detected by western blot and subclass specific Elisa. The Elisa was performed to gain a more detailed analysis of the antibody responses and a subclass specific Elisa was set up to delineate which murine immunoglobulin subclasses IgG1, IgG2a, IgG2b, and IgG3 were induced (Chapter 3, page 67-68)

APTT and Bethesda titre determination

The method described for APTT estimation is as described in chapter 3, page 64. For inhibitor titre estimation, Test plasma was serially diluted in imidazole buffer. 50 µl of test plasma (or diluted plasma) was incubated with normal mouse plasma for 2 hours at 37°C. Following the incubation 50µl of the mixture was added to 50µl FIX

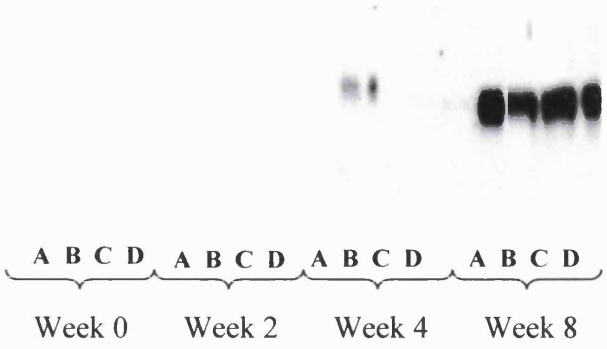
deficient plasma and incubated with 50µl APTT reagent. The APTT was recorded and the residual FIX was calculated by using a standard curve, which was generated from normal mouse plasma. The Bethesda titre was then calculated and adjusted according to the dilution of the test plasma.

Results

Haemophilia B mouse AAVmFIX injections

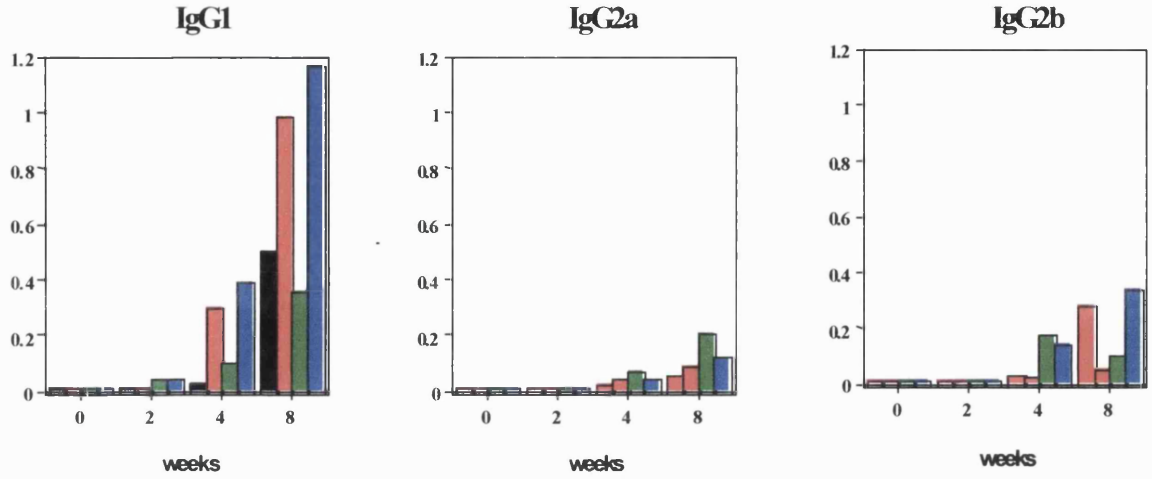
a) Western blot analyses shows the temporal pattern of antibody production following the injection of an AAV vector encoding murine factor FIX. Figure 53 shows the serum samples measured at 2, 4, 8 weeks post injection. The blots show no antibody detectable in the pre, 2 weeks samples. However by 4 weeks signal was detectable 2 out of the 4 mice and 8 weeks signal was clearly detectable in all 4 mice. (For method, see Chapter 3, page 76).

Fig 54 *Western Blot, Temporal pattern antibody formation*



b) In order to characterise the further the nature of the antibodies induced, the subclass specificity of these antibodies was determined by setting a subclass specific elisa as described in materials and methods (Chapter 3, page 67). The figure below shows the titres induced of IgG1, IgG2a, or IgG2b post injection

Figure 55 *Subclass Antibody induction following AAVmFIX injection*



The figure above shows the antibody subclasses after injection with an AAV vector encoding murine factor IX. The antibodies produced were almost exclusively of an IgG1 class in the injected animals, suggesting a Th2 driven humoral response was occurring.

Further CD4 Th cell subtype characterisation in antibody response

As discussed above in the introduction (p180) the presence of an IgG1 response in mice is associated with a CD4+T helper subset, which secretes the cytokines IL-4, IL-10 that activate B cells to produce IgG1.

A further experiment was designed to inject Knockout mice, deficient in $CD4^+$ cells or IL-4 and follow whether these animals made antibodies. Since the production of antibodies is a T cell dependent process, knockout mice deficient for CD4 would not be expected too produce antibodies. Similarly the production of antibodies in mice deficient in IL-4 may be expected to produce less or no antibodies (see figure 51,p180)

Aim

The aim of the experiments were to define if antibodies could be made in these strains (IL-4 KO, CD4 KO). If no antibodies were produced this would support the importance of CD4+, IL-4 participation in antibody production.

Methods

Both strains of mice bred (n=4) on a C57BL6 background were obtained from Jackson Laboratory (USA). These animals received IM injections at day 0 with the AAV vector as described above.

Results

a) *CD4 + Deficient mice*

Following administration of AAV vector, none of the CD4+ deficient mice (n=4) made any anti human FIX ab when assayed at 4 and 8 weeks post injection (data not shown). The result was identical for IgG1, IgG2a, IgG2b and IgG3 subclass confirming the T cell dependent nature of the anti hFIX after vector injection.

b) *IL-4 deficient mice*

IL-4 deficient mice failed to develop IgG1 anti hFIX following injection (n=4, assayed at 4 and 8 weeks post injection). They did show IgG2a/b responses *(data not shown*). Since Th2 proliferation is IL-4 dependent, these mice cannot develop Th2 help. These results demonstrate that that a part of anti hFIX production after AAV vector administration is due to activation of Th1 helper cells, although Th1 responses are low compared with adeno virus injected mice as demonstrated by cytokine release (Table 7,p159)

Assessment of FIX inhibitory activity of antibodies

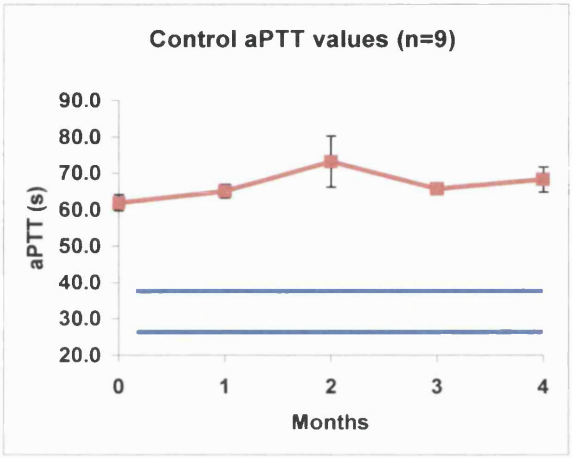
After the AAV injections, mouse serum was drawn at 2 weekly intervals for estimation of clotting times by APTT assay, as described in Chapter 3, page 64. Normal values were 60-80 seconds for a haemophilic mouse, and 26-36 seconds for a

normal mouse. In order to determine whether these antibodies were inhibitory or not, a Bethesda assay was carried out to quantitate the antibody titre (Chapter 3, page 64)

a) APTT values.

The graph below shows the values for APTT values after injection.

Fig 56 *APTT values of Haemophilia B injected animals*

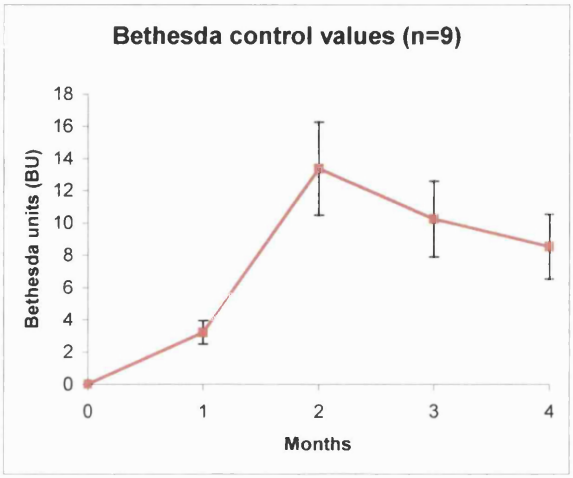


The figure shows that none of the injected mice corrected their APTT value after vector injection. The area between the two blue lines represents the normal APTT values for a mouse.

b) Bethesda Titre

Due to the failure of correction in APTT, the presence of inhibitory antibodies was tested by Bethesda assay. The results are shown in the graph below:

Figure 57 *Bethesda titres of Haemophilia B mice post injection*



As can be seen the Bethesda titre became maximal at about 2 months post injection. None of the injected mice showed correction in their APTT (range 60-80s) indicating factor IX inhibitory antibody activity. Although there was individual variation in the titre and temporal appearance of the antibodies by 1 month all animals had developed a positive Bethesda titre. The Bethesda titres ranged between 4-16 BU at 1 month and declined slightly over time but remained sustained at 6 moths post injection.

Summary

The data presented above show that none of the injected animals corrected their APTTs. All of the injected animals developed antibodies to murine factor IX as shown by both subclass specific Elisa and western blot. The antibodies were of predominantly of IgG1 subclass and inhibitory in nature as shown by Bethesda titre. These experiments demonstrated that these gene-deleted animals make antibodies to FIX as would be predicted by a protein-based approach. In this regard it appears that the result by the gene-based approach is no different from the protein-based approach.

7.5.2 Cellular Immune responses

All the above experiments described were designed to look for the occurrence of a humoral immune response. However it is possible (Chapter 1, pages 57-59) to elicit a cellular immune response using a gene based approach due to the intracellular production of proteins entering an MHC class I pathway.

Although a CTL assay for human factor IX was eventually devised (as described in Chapter 6), because of the unavailability of reagents at the outset of these studies it was not possible to study whether there was a CTL response in a murine model of haemophilia B. Therefore an experiment was designed to see whether there was evidence of expression of murine FIX in the injected muscle. This would at least provide indirect evidence of whether a CTL response was occurring in the transduced muscle. If a CTL response was occurring then should not be possible to detect FIX mRNA sequences since this would be destroyed by a CTL response. However, if no CTL response was present FIXmRNA should be detectable.

Experimental Design

The experiment was designed by injecting murine muscle as described above and looking for expression of factor IX expression by an RT PCR assay to look for the presence of factor IX mRNA. The aim of these experiments was to demonstrate whether evidence of Factor IX expression in muscle could be detected at 2 months post injection.

Method

2 haemophilia B animals were injected at day 0 with AAVmFIX at a dose of $4x10^{10}$ particles per animal. At 2 months post injection, the mice were sacrificed and the injected muscle was excised. Total RNA was isolated from muscle using Trizol reagent (Gibco/ BRL). RNA (1μg) was reverse transcribed using a kit from Gibco/ BRL. PCR primers specific for the mFIXcDNA (forward primer 5'-GATTGTAACTGCTGCCCACTGTCT-3' and reverse primer 5'-AATCTTTGCCTCCTTCCGGTAGC-3') were used to amplify 434-bp of the mFIX transcript using 1/5 of the cDNA product as the template (annealing temperature of 54°C, 35 amplification cycles). No amplification product was obtained from uninjected muscle or from RNA that was not reverse transcribed.

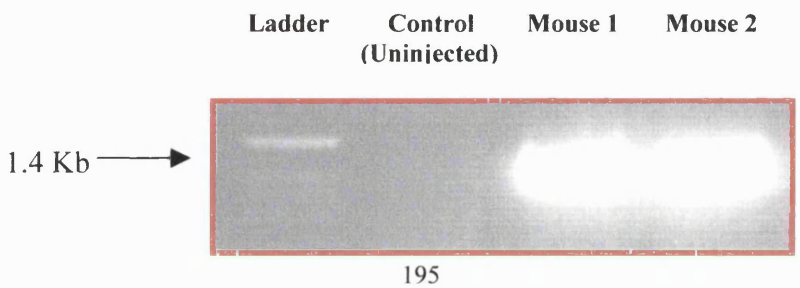
Vector

The vector used for these experiments is described in the previous section (page 181)

Results

Once the extracted mRNA was reversed transcribed, the DNA was run out on agarose gel as shown below:

Fig 58 *FIX DNA expression in injected muscle*



The figure shows a strong positive signal for the factor IX cDNA (size 1.4 Kb) illustrating that expression was still present in muscle at 2 months post injection. This study provided indirect evidence that there could not have been a CTL response directed against the injected muscle, since if there was, no expression of murine factor IX would have been demonstrated by RT-PCR.

Summary

The experiments performed shows that AAVmFIX intramuscular injection in haemophiliac animals does not appear to manifest a CTL immune response. This is an important result, since this property supports the use of these vectors in gene transfer protocols .The results also support the data described in chapter 6, where it was shown that AAVhFIX based transduction in muscle dose not elicit a CTL response.

7.6 Haemophilia B Treated with Immunomodulation

The results observed in the previous section show that all the injected animals produced an inhibitory antibody response by 2 months post vector injection. This situation presented here is different from the conventional protein-based approach for several reasons. Firstly, in the protein-based approach in Haemophilia B, there is a mixture of mutations leading to inhibitor formation, which includes both large gene deletions and point mutations. Most of the mutations result in point mutations i.e. *mis*-sense resulting in a circulating protein which although present in normal amounts is qualitatively abnormal. However in the animals treated in this gene based approach, the animals carry a large gene deletion in the first three coding exons affecting the promoter of the factor IX gene where no circulating factor IX antigen is produced. This represents the worst case scenario for provoking the development of inhibitors since there are no circulating epitopes, which may tolerise the animal to any future neo antigenic challenge.

An experimental strategy was designed to try and avert formation of inhibitors in a gene-based approach. The approach taken was to inject the gene-deleted animals with the vector and give them at or around the time of injection an injection schedule with

an immunomodulatory agent designed to suppress any antibody formation should it occur.

Use of Immunomodulation in a gene based approach

Although there have not been many studies addressing the blocking of an inhibitory response to the transgene product several studies have looked at immunomodulatory strategies in the context of viral vector readministration. Kay et al (1995) successfully used the agents CTLA-4 Ig and CD40L Ab combined to block both cellular and humoral responses to Adenoviral vectors to allow vector readministration.

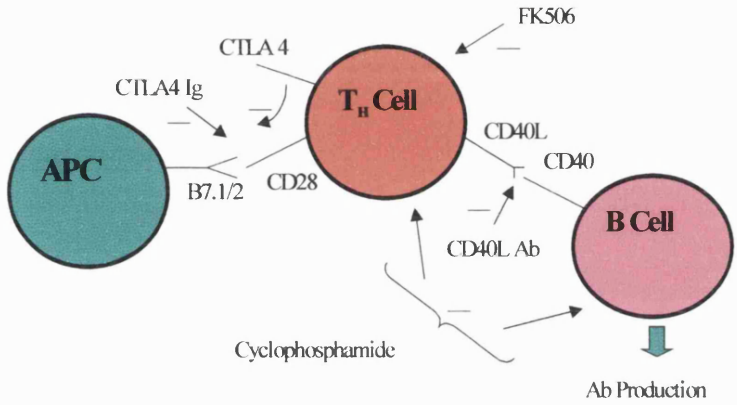
In order to block the immune response to the transgene product factor IX, it is necessary to disable the effector arm of the response. Since the development of inhibitor formation is a T cell dependent response, it will be necessary to eliminate or render the T cell component functionless. Immunologically, this means either clonally deleting the antigen specific T cell clones, or anergising them. This may be achieved by a host of different agents, which interfere or block the co stimulatory pathways involved in cognate T and B cell activation pathways.

The proposed agents for use in these studies is as follows

- Cyclophosphamide
- FK506
- CTLA4-Ig
- Cyclosporin A
- CD40 L Ab

The mechanisms of action of the various immunomodulatory agents tried in the following experiments are shown below and it can be seen that each agent has different mode of action.

Fig 59 *Immunomodulatory agents in T- B cognate cell interactions*



The overall aim of these strategies was to effectively disable the immune system at the time of antigen presentation. This would allow tolerance to occur to the *neo antigens* being introduced – in this case human factor IX. Several of these agents have been tried in other gene based protocols are described in detail below:

Cyclophosphamide

Cyclophosphamide belongs to the nitrogen mustard subclass of akylating agents. It has a wide application in the treatment of neplastic and autoimmune disorders. The drug is also used to suppress antibody formation in the setting of acquired FVIII inhibitors, or those arising in the setting of haemophilia (as part of the Malmö tolerance induction regimen). It works by inserting into the DNA helix and becoming cross-linked leading to disruption of the DNA chain. It is especially effective against rapidly dividing cells and is therefore sometimes given at the time of grafting to block T cell proliferation. Cyclophosphamide in these studies will inhibit the both B and T cell pathways in antigen presentation of factor IX.

Use in gene therapy

Liver and Lung

In a gene-based approach cyclophosphamide has been used to prolong transgene expression following administration with an Adenoviral vector in both mouse liver and lung. (Jooss *et al.*, 1996), it appeared to do this by blocking activation and mobilisation of CD4+ and CD8+ cells. As a result transgene expression was prolonged with reduction of inflammation and using higher doses of cyclophosphamide formation of neutralising antibodies was prevented allowing further readministration of the vector. Slightly different results were obtained in the lung where it was found that only at the highest dose was it possible to prolong transgene expression whereas at all doses low to high it was possible to prevent the formation of neutralising antibodies. These experiments pointed out the importance of target tissue variability in the outcome of prolonged gene expression.

Muscle

Other investigators have looked at the use of cylophosphamide in blocking the response to human factor IX secretion from an adenoviral vector. The experiments were carried via a muscle directed approach in Adult Swiss Webster mice. The investigators attempted to tolerise the host to adenoviral and FIX protein, using Cyclosporin A (CyA) and cyclophosphamide (CyP) (Dai *et al.*, 1995) in the hope that CyA would block cell mediated immunity, while cyclophosphamide would act by killing rapidly dividing cells involved in immune activation.

Using this approach it was found that CyA alone had no effect while CyP alone and in conjunction with CyA prolonged expression. The regimen of cylophosphamide administered was 50mg/kg every 2 weeks for 8 weeks. In the animals treated with cyclophosphamide, all animals showed elevation in plasma factor IX levels and one animal was completely tolerised. Although only one animal was completely tolerised to it did point out that this strategy was possible to achieve tolerance, but more numbers will be required to reproduce these results.

FK506

Lochmuller et al (Lochmuller *et al.*, 1996), demonstrated that following Adenoviral (Ad) vector encoding a dystrophin transgene, use of the immunosupressive agent FK506 over 5,10,30 and 60 days after Ad mediated gene transfer resulted in maintenance of the initial transgene expression for at least 2 months even when FK506 was discontinued after 1 month. This was in keeping with a marked reduction in the inflammatory infiltrates observed at the injection sites in muscle. Additionally it was found that FK506 efficiently suppressed the humoral immune response against both the vector proteins and the transgene product dystrophin. They concluded that the initial sensitisation of the immune system by the antigenic load triggered a humoral and cellular immune response which could be significantly subverted by relatively short term immune suppressive treatment.

Cylosporin A

Cylosporin A (CyA), FK506 are fungal metabolites with potent immunosuppressive properties. Although chemically unrelated both drugs have similar mechanisms of action. They block the activation of resting T cells by inhibiting the transcription of genes encoding IL-2 and the high affinity IL-2 receptor, which are essential for T cell activation. The drugs exert their actions by binding to cytoplasmic proteins called immunophilins forming a complex that block phosphatase activity of calcineurin. This prevents the formation and nuclear translocation of the cytoplasmic subunit NF-ATc and its subsequent assembly into HF-AT a DNA binding protein necessary for transcription of the genes encoding IL-2 and IL-2R. The drugs therefore inhibit Th cell proliferation, Th cytokine expression, and reduce subsequent activation of various effector populations involved in graft rejection.

CD40-CD40L interactions

The activated CD4+ T cell expresses a protein on its surface CD40 ligand that engages CD40 on the surface of the B cell. Ligation of CD40 and the interaction of antigen with surface immunoglobulin are sufficient to induce robust B proliferation and to enhance the amount and affinity of antibody produced and to allow the B cell

to switch from IgM to IgA, IgG, or IgE production. (Clark & Ledbetter, 1994; Foy *et al.*, 1994a; Foy *et al.*, 1994b). The importance of the pivotal role of CD40-CD40L in B cell activation and isotype switching comes from the studies of X linked hyper immune syndrome, which is characterised by elevated IgM levels and the absence of other isotypes. The defect results from mutations in the CD40L gene.

The importance of the CD40-CD40L interaction has led to the development of a hamster monoclonal antibody (MR1) directed against murine CD40 L, which *in vitro* blocks T and B cell proliferation (Noelle & Snow, 1992; Noelle *et al.*, 1992a; Noelle *et al.*, 1992b). When used *in vivo* it is also able to inhibit primary and secondary antibody responses to TD (Thymus dependent) antigens SRBC and TNP –KLH. (Foy *et al.*, 1994b). The basis of these actions of this antibody forms the rationale for its use in blocking antibody formation in the experiments described later in this chapter.

7.6.2 Experimental protocol to study immunomodulatory approach in a gene based situation

Aim

The aim of these experiments was to try and block inhibitor formation in a gene based approach. The experimental design taken was to follow the protocol as described above and inject haemophilia B mice with an AAV encoding murine factor IX, but in addition to combine this the use of an immunomodulatory agent given at or around the time of vector injection.

Method

8-week-old haemophilia B mice were injected at day 0 with AAVmFIX at a dose of 4 $x10^{10}$ vector genomes per Kg. Around the time of injection, immunomodulation therapy was administered to the mice according to the schedules outlined in the following table. The injected mice were then bled at 2 weekly, and then at monthly intervals to monitor for the development of inhibitors by plasma based assays (Elisa, Western Blot) and functional based clotting assays (APTT, Bethesda assay). The scheduling and dosing of the various immunomodulatory agents used in the following experiments is shown in below:

- *CD40 L Antibody*

0.1mg I/P at d –3, d0, d+3, d+6, d+9

- *Cyclosporin A*

100 mg/kg I/P 3 times weekly

- *Cyclophosphamide*

50mg/kg I/P at d0, week 2, week 4, week 6

- *FK506*

125µg S/C Alternate days from d 0

- *CTLA- 4 Ig*

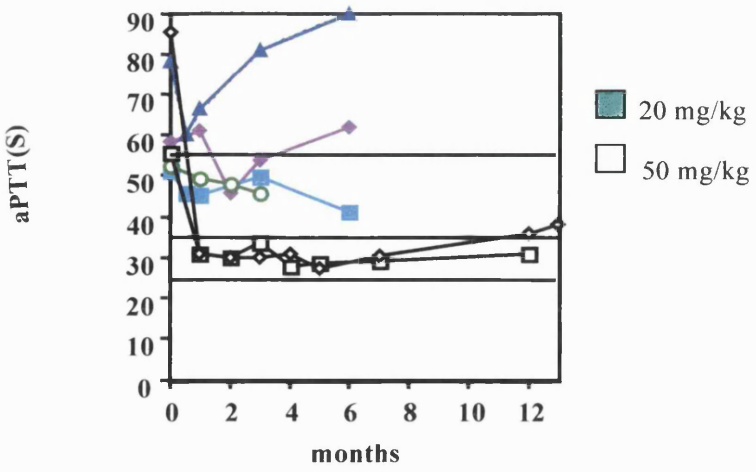
0.1mg I/P at d-3, d0, d+3, d+6, d+9

7.6.3 Results

a) *Cyclophosphamide Ab treated groups*

The drug was given at a dose of 20 or 50 mg/kg on a biweekly schedule starting from the day of vector administration ($1x10^{11}$ vg/mouse) up to 6 weeks after gene transfer. As shown in the figure below, the combination of vector administration and repeat-injections of cyclophosphamide gave a partial to complete correction of the APTT in 4/6 mice. Two mice, which had received cyclophosphamide at only 20 mg/kg, formed inhibitory antibodies with a Bethesda titre of 3-7 BU, while the other animals treated did not form inhibitors. Expression of mFIX in those mice (n=2) that showed complete correction of the APTT which was sustained for the duration of the experiment (>6 months) indicating that transient immune suppression was sufficient to obtain long-term expression without induction of an immune response

Figure 60 *Cyclophosphamide treated mice*



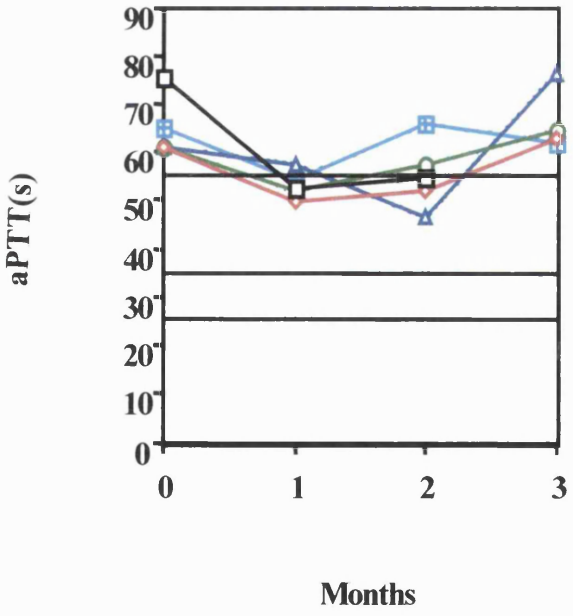
Cyclophosphamide (20 mg/kg [filled symbols] or 50 mg/kg [open symbols] given by intraperitoneal injection very two weeks starting from the day of vector administration up to week 6).

As can be seen from the above figure the administration of cyclophosphamide suppresses antibody formation in two of the injected animals, and did show some partial correction in the other injected animals.

b) *CD40L Ab treated*

Administration of anti-CD40L starting three days before injection of vector up to day 9 after gene transfer resulted in partial correction of the aPTT in 3/5 mice during the first two months of the experiment. By the third month, aPTT values had returned to pre-treatment values, and inhibitory antibodies were measured in 4/5 mice by Bethesda assay (≤10 Bethesda units). These data indicate a marginal impact of anti-CD40L administration on the anti-mFIX immune response.

Figure 61 *anti CD40L Ab treated mice*



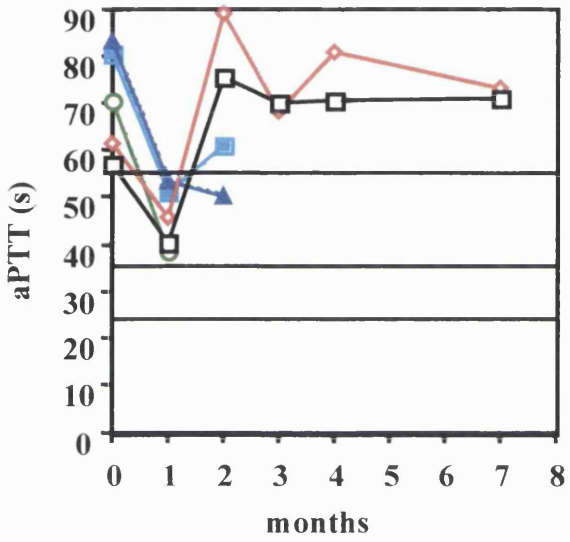
Mice treated with anti-CD40L (intraperitoneal, 0.1 mg in 200 µl sterile saline on days –3, 0, 3, 6, and 9 after injection of vector).

c) *FK506 treated*

Treatment with FK506 invariably resulted in partial correction of the aPTT (5/5 mice) at one month after IM injection of AAV vector. However, systemic mFIX expression was subsequently lost, and low titer inhibitory antibodies ≤3 BU were measured at 3-4 months. FK506 was effective in blocking the antibody formation against mFIX as long as the drug was administered (up to day 30 on every alternate day). This effect

did not persist once administration of the drug was discontinued, thus resulting in a delayed and weakened immune response, which was, however, sufficient to prevent efficacy of treatment by gene transfer. Additionally, several animals developed infections as a result of the prolonged immune suppression.

Figure 62 *FK506 treated mice*



Mice that received FK506 (subcutaneous, 125 μg every alternate day, starting at the day of vector administration, for up to 1 month)

c) *Cyclosporin A*

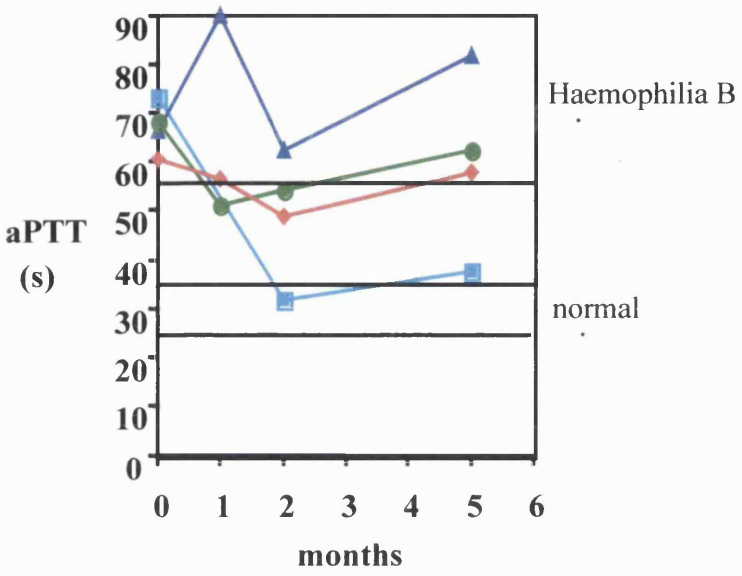
9 mice were injected with concomitant cylosporin A. These animals showed substantial renal toxicity and often died within the first month of treatment. Those animals (n=3) that survived showed no signs of correction of their coagulation times despite reduced or undetectable inihibitor titres (*data not shown*).

e) *CTLA-4 Ig treated*

Mice were treated with CTLA4-Ig on an identical schedule and showed partial correction of their APTT (3/4 mice) during the first two months, but APTT levels returned to baseline by the third month in 3/4 mice. Two mice developed a low titer inhibitory anti-mFIX (3-5 Bethesda units at a 5 month time point), while one animal continued to express mFIX for at least 5 months with no detectable Bethesda titer (*data not shown*). These data show that transient immune suppression combined with

gene transfer can result in sustained mFIX expression and partial correction of the haemophilic phenotype.

Figure 63 *anti CTLA-Ig treated mice*



Mice that received CTLA4-Ig (intraperitoneal, 0.1 mg in 200 µl sterile saline on days –3, 0, 3, 6, and 9 after injection of vector)

7.7 Discussion

Data from the haemophilia B database suggest that increased loss of coding information, as occurs with an early stop codon or a deletion in the FIX gene, may increase the risk of inhibitor formation in protein-based replacement therapy.

It may be predicted therefore, that in the setting of gene transfer, similar results may be obtained. Use of a human transgene in a gene-based setting prevents a straightforward interpretation of data because of the occurrence of a humoral response to the non-species specific transgene product. A relative advantage of haemophilia B as a paradigm disease for the treatment of this genetic disorder by gene therapy is the availability of small and large animal models of the disease. The cloning of murine factor IX and its incorporation into a construct encoding this transgene facilitates a more accurate analysis of immune responses in the homologous murine haemophilia B animal model. The mice selected for these studies carry a large gene deletion without detectable mFIX transcript or antigen, and they represent perhaps the most stringent test in terms of lack of tolerance to the protein product of the donated gene.

The experiments described in this chapter were designed to characterise the formation of inhibitory antibodies in a gene-based approach for haemophilia B using an AAV vector. All of the injected mice formed inhibitory antibodies by 2 months. The results echo the situation for a protein-based approach where possession of a large gene deletion correlates with an increased likelihood of inhibitor development.

The antibodies produced in these experiments were of an IgG1 subclass in keeping with a Th2 driven T helper cell response and were inhibitory in nature as evidenced by the raised Bethesda inhibitor titres. In order to carry out these studies further to define risk, it will be necessary to test out these injections in animals carrying different mutations i.e. point mutation s to see if this results in a lower incidence of inhibitor formation. Some of these questions will be addressed in chapter 8.

Although the injections in these gene deleted mice generated the production of inhibitory antibodies no evidence of cellular destruction (CTL response) of the injected muscle was demonstrated as evidenced by the long-term persistence of factor IX mRNA.

These studies demonstrated that inhibitory antibodies are made to the transgene product human factor IX and in keeping with the protein based situation. Therefore it is important to look at strategies, which can overcome this response.

It is known from replacement therapies, that immune tolerance regimens have been used to treat already established inhibitor responses. In these treatments, clotting factors are given on a prolonged daily basis in high dosage (+/- immune suppression) to achieve tolerance although the out come in these cases is not always predictable. It may be argued therefore, that a process where clotting factors are continually produced such as a gene-based appraoch, may itself be tolerogenic and used a therapy for the treatment of inhibitor development (Evans & Morgan, 1998).

A number of immunomodulatory strategies were devised to test whether immunomodulation given at or around the time of vector administration could avert inhibitor development. It should be emphasised that is a different challenge to the protein-based approach, where clinicians usually try to avert inhibitor formation once

it has occurred. In the approach described in these experiments the aim was to try and block inhibitor formation before it occurred.

Transient immune suppression has been evaluated in a number of studies as a tool to prevent formation of neutralising antibodies against viral vector particles or to reduce cellular immune responses and inflammation typically seen with early generation adenoviral vectors. However, little is known about the effect of such immune modulation on a humoral immune response against a secreted transgene product such as FIX. Activation of T helper cells and B cells following antigen presentation by APCs is not only dependent on presentation of peptides (T cell epitopes) by MHC class II molecules and binding to T cell receptors, but also requires the presence of co-stimulatory signals to achieve full activation. These co stimulatory signals include the interaction of CD40 and CD40 ligand (CD40L), an important signalling pathway required for B and T cell activation (Kay *et al.*, 1997; Wilson *et al.*, 1998), and the B7-CD28 co-stimulatory pathway. The former pathway can be specifically blocked by anti-CD40L antibody, the second by soluble CTLA4-Ig fusion protein or anti-B7.1/2 antibodies (Linsley, 1995a; Linsley, 1995b; Linsley *et al.*, 1995; Linsley & Golstein, 1996).

Both strategies have been investigated as a tool for prevention of humoral and cellular immune responses against viral gene transfer vectors (Kay *et al.*, 1997). It is known that efficient antigen presentation in the absence of the necessary co-stimulatory signals will result in antigen-specific T cell anergy, i.e. subsequent inability of these T cell clones to initiate an immune response against the antigen that they recognise.

In the experiments presented in this chapter the aim was try and prevent the formation of inhibitory antibodies by interfering with activation of effector CD4 cells and subsequent proliferation of antibody producing B cells. Several agents were tested including both antibodies (anti CD40, CTLA-4 Ig) and drugs (Cyclophosphamide, FK506, Cyclosporin). These agents have been previously been studied in the context of gene therapy as shown in the following table:

Table 9 *Immunomodulatory agents*

Agent	Target Tissue	Reference
Cyclophosphamide	Lung	Jooss et al
Cyclophosphamide	Liver, Lung	(Jooss *et al.*, 1996)
Cyclophosphamide	Muscle	(Dai *et al.*, 1995)
FK506	Muscle	(Lochmuller *et al.*, 1995; Lochmuller *et al.*, 1996)
CTLA-4	Liver, Lung	(Kay *et al.*, 1995),(Jooss *et al.*, 1998b)
CD40L Ab	Liver and Lung	(Yang *et al.*, 1996c)
CD40L Ab/ CTLA-4	Liver	(Kay *et al.*, 1997)
Cyclosporin A	Muscle	(Dai *et al.*, 1995)

The formation of inhibitory anti-mFIX was somewhat weakened and delayed in mice treated with anti-CD40L, yet overall the effect was marginal, and mice still developed inhibitors. Better results were obtained with CTLA4-Ig. Only 2/4 mice developed (low titre) inhibitors, and 1/4 animals showed sustained correction of the APTT coagulation time. CTLA4-Ig has been successfully used to prevent rejection of transplants in experimental models (Linsley, 1995a; Linsley *et al.*, 1995; Linsley *et al.*, 1996; Linsley & Golstein, 1996) and has also been tested for prevention of inhibitors against human FVIII protein infused intravenously into haemophilia A mice. In this study, antibody formation could only be prevented if CTLA4-Ig was given at the same time as the FVIII antigen.

In the work described here, reduced inhibitor titres were seen in all mice treated with CTLA4-Ig that resulted in transient partial correction of the haemophilia B phenotype as a result of prolonged transgene expression. Moreover, one animal showed sustained correction (for several months after vector injection) without inhibitor formation. This result suggested that it was possible to achieve long-term expression by transient blockage of a co-stimulatory pathway.

Optimisation of the dosing and schedule with administration of CTLA4-Ig may result in a greater success rate with this approach. A disappointing result was obtained with the combined use of CD40 ligand antibody and CTLA- 4Ig and it is unclear why this was so. Previous experience with these two agents in a gene-based approach has yielded considerable success in vector readministration experiments (Kay *et al.*, 1995), (Halbert *et al.*, 1998), (Kay *et al.*, 1997). It may be that simple adjustments to the amount and timing of dosing of this approach may yield better results. This type of dosing experiments will need to be done in the future to test this hypothesis

In addition to blocking costimulatory pathways, tolerance may be induced by the use of specific drugs with immunemodulatory properties. Dai (Dai *et al.*, 1995) and colleagues have previously shown that tolerisation could be affected by the use of the immune suppressive drug cyclophosphamide in the context adenoviral gene transfer encoding the canine transgene for factor IX in a murine model. There is a wide range of immunomodulatory drugs available for use in the clinical setting, and several of these drugs were tried out to avert inhibitor formation in a gene based approach. These agents work either by blocking differentiation and/or proliferation of B and T lymphocytes (Cyclosporin and FK506) or by clonal deletion of antigen specific T cells.

The agent FK506 (Tacrolimus) blocks intracellular signalling pathways, and prevents T cell activation. The drug has been shown to reduce cellular and humoral immune responses and is successfully used to prevent rejection of organ transplants in the clinical setting (prevention of allograft rejection, graft versus host disease and others). Equally FK506 has been used successfully in gene transfer experiments to block cellular and humoral responses to Adenoviral gene transfer encoding the mini dystrophin gene in the *mdx* mouse model of Duchenne muscular dystrophy (Lochmuller *et al.*, 1995; Lochmuller *et al.*, 1996). When tested in these experiments, consistent administration of FK506 resulted in marked reduction of APTTs without measurable anti-mFIX responses in all of the treated mice. However, correction of the haemophilic phenotype was lost after discontinuation of the drug, and low-titre inhibitors emerged at later time points indicating that induction of tolerance to mFIX with this treatment was unsuccessful. When the drug was given continuously

correction of the haemophilic phenotype was achievable. Therefore, if long-term administration were attainable in a non-toxic fashion, this regimen holds considerable promise.

Cyclosporin a widely used immune suppressant in the field of clinical renal and bone marrow transplantation induced strong toxicity when administered to the mice at the initial dose of 100mg/kg. Subsequent lowering of the dose to non-toxic levels did not give evidence of systemic mFIX expression following gene transfer and therefore the studies with this agent were abandoned. The results with this agent were a little disappointing, and perhaps in future studies consideration could be given to use of this agent as an adjunct to treatment with other agents.

In contrast, cyclophosphamide, a drug that is cytotoxic to proliferating cells such as activated B or T cells, was effective at a dose of 50 mg/kg (3/3 mice had correction of coagulation times) and partially effective at 20 mg/kg (1/3 mice) in blocking an immune response that could prevent sustained systemic expression of mFIX. In two haemophilia B mice, the combination of muscle-directed gene transfer and transient immune suppression with cyclophosphamide achieved sustained complete correction of the APTT. The mode of action of cyclophosphamide in the setting of bone marrow transplants is thought to be a combination of clonal deletion of antigen-specific lymphocytes and induction of T cell anergy.

In this gene based approach cyclophosphamide probably works via a similar action. The success of cyclophosphamide in these studies confirms results seen in other gene based therapies where this agent successfully suppressed immune responses either in the setting vector readministration or primary immune response to the vector or transgene.

Summary

From these studies, it appears that FK506 and Cyclophosphamide are promising agents in suppressing inhibitor formation (Fields *et al.*, 2001b). More importantly, it seems that the overriding principle of transient immune suppression given around the time of vector administration may be useful in suppressing inhibitor formation in a gene based approach(Fields *et al.*, 2001b). However, the use of agents such as cyclophosphamide in humans is a contentious issue, since the use of this agent brings with it concerns over infertility induction and the long-term risk of insertional mutagenesis and oncogenicity. Further studies will be required to try and optimise these strategies, and also to test out new agents as they become available as more knowledge accrues about the mechanism(s) involved in the induction of T cell tolerance and co stimulatory pathways.

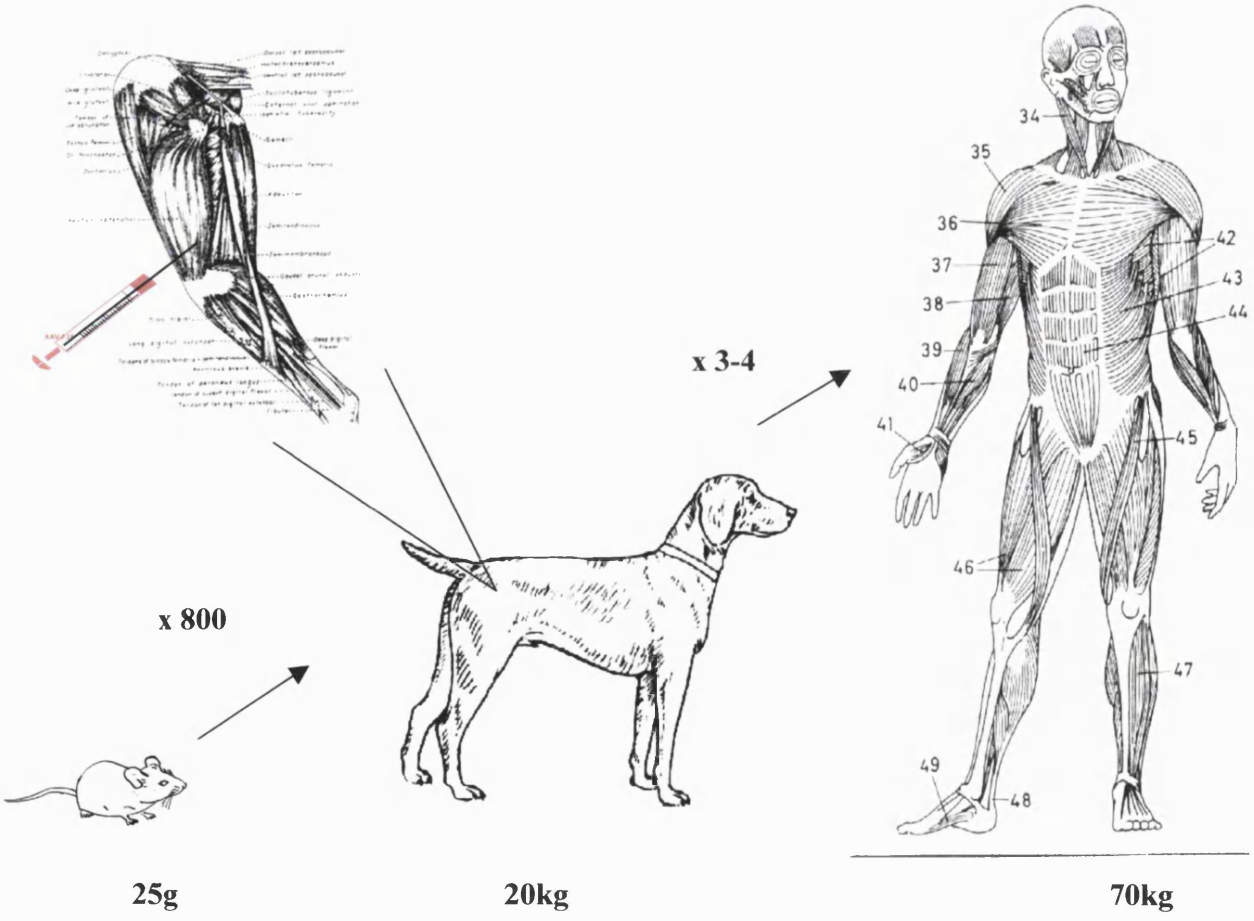
CHAPTER 8

GENE TRANSFER IN A LARGE ANIMAL MODEL OF HAEMOPHILIA B

8.1 Introduction

One of the major requirements of any gene therapy strategy is the successful application of a proof of principle strategy in a large animal model before proceeding to human subjects. In haemophilia, there are large animal models for both haemophilia A and haemophilia B and this affords the opportunity for pre clinical studies. The existence of large animal models has proved invaluable in permitting pre clinical studies to test the feasibility of scaling up to determine if the promising results gained from the mouse studies could be reproduced in dogs. This is not a trivial exercise and represents a scale up of 400 (*Figure 63*). One of the main problems encountered is the extra vector amount that is required to produce the equivalent dose in a dog.

Figure 64 *Scale up in animal models*



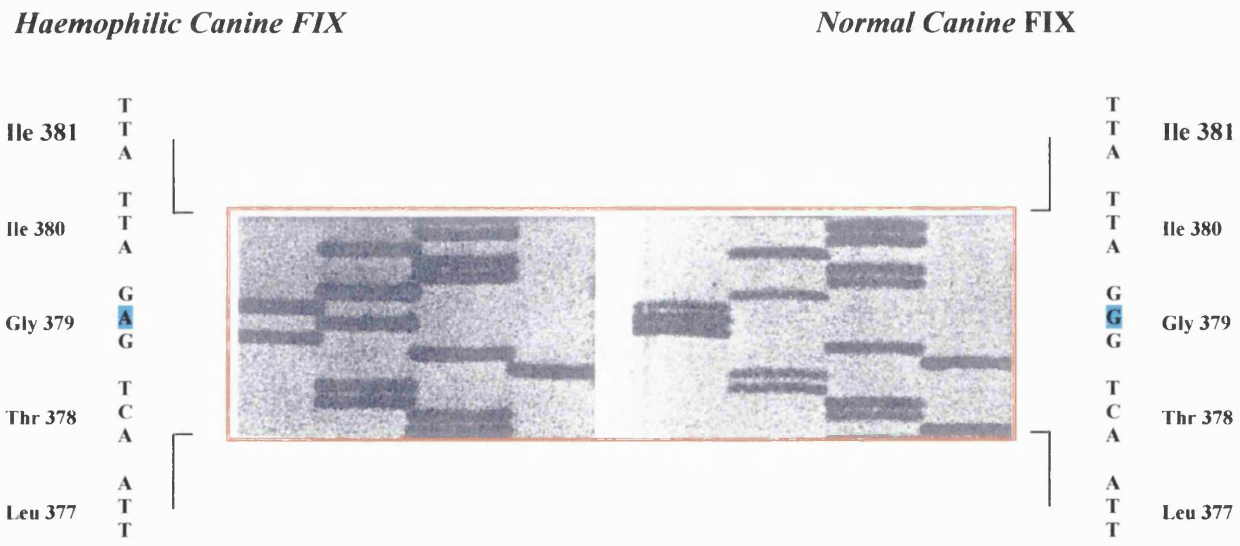
8.2 Canine Models of Haemophilia

Three canine models of haemophilia B have been described and are outlined in the following table.

Canine Model	Mutation	Reference
Chapel Hill(USA)	Mis-sense [G ⟶ A nt1477]	Evans et al(1989)
Aubern (USA)	Small deletion Exons	Mauser et al (1996)
Cornell (USA)	Complete deletion	Brooks et al(1997)

The one selected for the studies in this chapter is the colony kept at Chapel Hill, North Carolina United States. The genetic defect in the dog colony is a mis-sense mutation at a highly conserved glycine residue in the part of the gene encoding the catalytic domain (Evans *et al.*, 1989).

Fig 65 *Canine mutation*



This defect results in a FIX transcript being produced, but with FIX activity and antigen levels of less than 1%. Molecular modelling suggests that the mutant protein is unstable because it cannot fold properly (Evans *et al.*, 1989). The absence of the protein in the circulation suggests that the mutant protein is destroyed intracellularly, but importantly the levels achieved may be enough to induce T cell tolerance to the

protein (Evans *et al.*, 1989). It is important to note that the dogs used in the study carry a *different* mutation to the mice (Chapter 7) that carry a large gene deletion resulting in no mRNA transcripts and no protein production intracellularly. Generally, these dogs do not develop inhibitors in response to treatment with canine plasma. Two other mutations have been described, the first by Mauser et al(Mauser *et al.*, 1996) in which dogs maintained at the university of Auburn carry a small deletion in the portion of the gene encoding the activation peptide. FIX transcripts are barely detectable on northern blot. A third colony with haemophilia B has been also been described with a complete deletion of the factor IX gene (Brooks *et al.*, 1997).

8.3 Gene Therapy In Canine Models of Haemophilia B

Despite a lot of preclinical work in mouse models, only one reported study has been able to report sustained expression of factor IX in a canine model of haemophilia B where a retroviral vector was used in a liver directed route (Kay *et al.*, 1994). In the studies conducted, the plasma levels achieved were low at 2-4 ng/ml (sub therapeutic) and also required several invasive procedures such as portal vein cannulation and partial hepatectomy. This approach would not be practical and unappealing in humans.

Intramuscular Gene Transfer in Haemophilia B

The studies described in dogs were designed to question whether the early promising pre clinical data on mice could be translated into a large animal model. The experimental design of the experiment was an ongoing dose escalation study in which the canines received AAVcFIX at day 0.

Aim

The aims of the studies were to look at immune responses using an intramuscular approach in a large animal model of Haemophilia B. The remainder of chapter is divided up into first an analysis of the humoral responses and then of the cellular responses.

Humoral responses

Humoral responses were analysed to the transgene product canine factor IX (cFIX). In order to study humoral responses knowledge of canine immunoglobulin subclasses is required.

8.3.1 Canine Immunoglobulin subclasses

Subclasses of IgG have been well described in man and other species with particular functional characteristics have being ascribed to them. These variants are well described in man and four subclasses IgG1 IgG 2 IgG3 IgG 4 are defined on the basis of their concentrations in the serum of normal individuals and their biochemical properties, such as electrophoretic mobility (Schur, 1987). The subclasses are functionally different. For example IgG1 and IgG3 bind most effectively to the $F_c\gamma R$ receptor of mononuclear cells and IgG3 is the most efficient at complement fixation via the classical pathway. The expression of specific subclasses in different immune responses is probably related to factors such as the nature of the stimulating antigen and the local presence of specific cytokines, which regulate isotype switching of the immunoglobulin subclasses. For example human IgG1 and IgG3 subclasses predominate in the immune response to protein antigens and may be induced by interleukin 10, whereas chronic stimulation with a protein antigen results in a predominance of IgG4 which maybe induced by interleukin 4 and interleukin 13 (Briere *et al.*, 1994). Carbohydrate antigens preferentially induce IgG2 antibodies (Barrett & Ayoub, 1986) and the expression of this subclass may be up regulated by interferon γ. (Kawano et al 1994)

In the canine species, four subclasses of canine IgG have also been isolated and a panel of monoclonal antibodies have been defined which can specifically identify each subclass in serological or immunohistochemical assays (Mazza *et al.*, 1993; Mazza *et al.*, 1994a; Mazza *et al.*, 1994b). This panel of monoclonal antibodies have been used to identify the canine subclasses involved in the pathogenesis of autoimmune disease in dogs; in particular autoimmune haemolytic anaemia where the predominant antibody was shown to be of the IgG1 subclass. In a further study carried out in animals with hypothyroidism the dominant subclasses of antibody found were

IgG1, IgG3, and IgG4 (Day & Mazza, 1995). With respect to the development of an inhibitory response to infused clotting factor protein in dogs, there is very little documentation of these responses in the literature. In a large animal species such as dogs, one might speculate that a humoral driven response occurs, resulting in a Th2 driven response since the antibody characteristically seen in humans is an IgG4 subclass.

In the series of studies performed in haemophiliac dogs, plasma samples were analysed for the presence of antibodies and their subclass determined by using anti dog IgG1 or IgG2 polyclonal antibodies. These antibodies were used to study antibody subclasses in dogs infected with leishmania. Both IgG1 and IgG2 were detected in sera from infected dogs but the IgG2 subclass was the dominant type involved.

Cellular responses

A T cell proliferation assay was set-up to look at the recall responses of circulating T cells to canine factor IX. Additional experiments were then set up to look at the cytokine profiles expressed by the proliferating lymphocytes in the injected dogs. This was performed by setting up an RT-PCR assay to look at intracellular cytokine expression of the proliferating cells.

8.4 Canine Experimental Protocol

Method

For the gene transfer experiments, an AAV vector encoding canine factor IX was injected intramuscularly into the haemophilia B canine model described above. A total of 7 dogs over a period of two years were injected with the AAV construct over a range of 8-60 intramuscular sites. Carbon particles were added to some aliquots of vector to facilitate identification of injection sites for later histological evaluation.

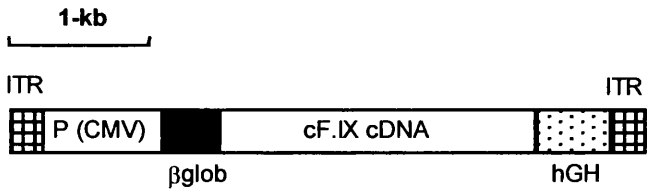
In performing this study, the following parameters were analysed, the production of canine factor IX antigen, clotting functional based assays the APTT and WBCT

(Whole blood clotting time). The presence of antibodies was examined by both Elisa and Western blot. To assess whether these antibodies were inhibitory or not, a Bethesda assay was performed.

The WBCT for a normal dog is 6-8 minutes and for a haemophilic dog is >60 minutes. In these studies a critical evaluation was made to look for the development of inhibitory antibodies during the course of the study. Additional further characterisation of the humoral antibody profiles was performed by using a subtype specific elisa was run with a polyclonal antibody to both IgG1 and IgG2 canine subtypes (Bethyl, US)(see chapter 3, page 73).

Vector

The construct used for the injections was prepared in a triple transfection method (Adenoviral free, see chapter 4,page 96) as described by (Matsushita *et al.*, 1998).
The recombinant AAV vector is shown as follows



The vector contains AAV inverted terminal repeats ITRs flanking an expression cassette containing the following. A) A cytomegalovirus immediate early enhancer / promotor, CMV splice donor/ β – globin gene splice acceptor (β globin) , canine factor IX cDNA (cFIX) up to the *Eco*RI site at nucleotide position 2565 (Evans *et al.*, 1989), and human growth hormone polyadenylation signal (hGH). The vector produced for these injections was made in an adenoviral free system (Nakai *et al.*, 1999). Briefly, 293 cells were co transfected three plasmids, pAAV–CMV-cFIX, a second plasmid (pHLP19) providing the AAV replication and capsid functions, and a third plasmid (pLadeno5) providing the adenovirus helper functions, E2a, E4, and VA. Cells were detached from the culture flasks with an EDTA solution 72 h post-transfection pelleted by centrifugation and lysed by repeated freeze/thaw. Recombinant AAV was purified by repeated CsCl density gradient centrifugation

(Fisher *et al.*, 1996b). The vector was titered by slot blot hybridisation on DNA extracts from viral particles.

Transient transfection of 293 cells in the presence of vitamin K (6 μg/ml medium) was used to show that the cFIX vector directs expression and secretion of functional canine FIX. Conditioned medium from AAV-cFIX transduced cells corrects the APTT of human FIX deficient plasma whereas conditioned medium from AAV-lacZ-transduced cells did not (*data not shown*). The total doses and dose per kilogram for each animal is shown in the table below.

Table 10 *Vector Dosage schedule*

Dog	Vector Dose/ Site	Vector Dose/Kg	Total Vector Dose
1(B45)	$4.4x10^{10}$	$1.4x10^{11}$	$8x10^{11}$
2(B46)	$1.25x10^{12}$	$1.1x10^{12}$	$1x10^{13}$
3(B48)	$1.1x10^{12}$	$3.4x10^{12}$	$6.7x10^{13}$
4(B93)	$1.4x10^{12}$	$2.9x10^{12}$	$3.9x10^{13}$
5(B85)	$2.1x10^{12}$	$8.5x10^{12}$	$5x10^{13}$
6(D31)	$2x10^{12}$	$8.4x10^{12}$	$3.2x\ 10^{13}$
7(B14)	$1.2x10^{13}$	$1.1x10^{13}$	$2.3x10^{14}$

Results

The results for all the injected dogs is summarised in the table below. The individual dogs are then discussed in detail.

Table 11 *Results*

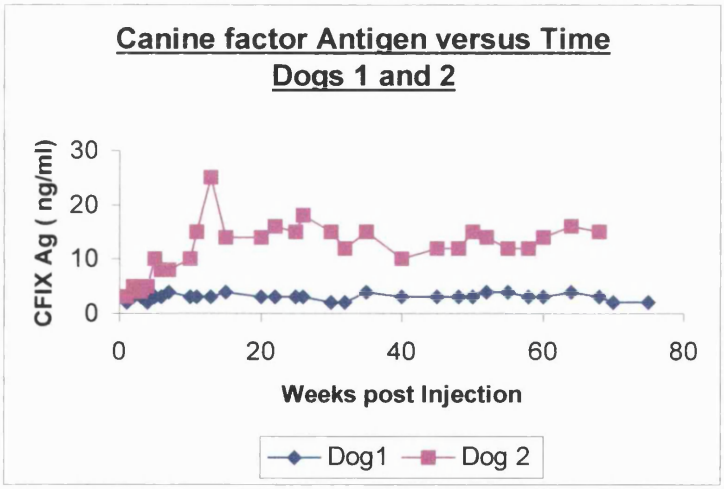
Dog	Vector Dose/ Site	Vector Dose/Kg	Total Vector Dose	Anti canine Factor IX	Inhibitory Antibody titre (*BU)	Duration of Inhibitor
1(B45)	$4.4x10^{10}$	$1.4x10^{11}$	$8x10^{11}$	None	None	-
2(B46)	$1.25x10^{12}$	$1.1x10^{12}$	$1x10^{13}$	None	None	-
3(B48)	$1.1x10^{12}$	$3.4x10^{12}$	$6.7x10^{13}$	IgG2	None	-
4(B93)	$1.4x10^{12}$	$2.9x10^{12}$	$3.9x10^{13}$	None	None	-
5(B85)	$2.1x10^{12}$	$8.5x10^{12}$	$5x10^{13}$	IgG1, IgG2	< 6.8 BU	8 Weeks
6(D31)	$2x10^{12}$	$8.4x10^{12}$	$3.2x\ 10^{13}$	IgG2	None	-
7(B14)	$1.2x10^{13}$	$1.1x10^{13}$	$2.3x10^{14}$	IgG1, IgG2	< 24.5 BU	2 Years

Dogs 1 and 2

The first dog injected was at a dose of 1.4 x 10^{11} particles/kg. Predicted from the early mice studies this should result in a level of expression of approximately 2.5ng/ml. The canine factor IX level measured by Elisa was in the range 2-3 ng /ml.(Fig 1). This dog displayed no evidence of antibody or inhibitor formation as determined by Western blot, Elisa and inhibitor screen. A second dog was then injected with a higher dose of vector (10-fold increase, 1.1 x 10^{12} vector particles/ kg. Again partial correction of the WBCT was seen and plasma levels of 10- 15ng/ml canine factor IX were reached. This was not enough to cause a correction in the APTT.

(Low levels of antigen cause WBCT correction before correction of the APTT, which requires higher antigen levels to achieve correction). No inhibitor was demonstrated by Bethesda screening and no anti cFIX antibodies were detectable by Western immunoblot.

Figure 66 *Canine FIX Antigen levels Dogs 1&2*

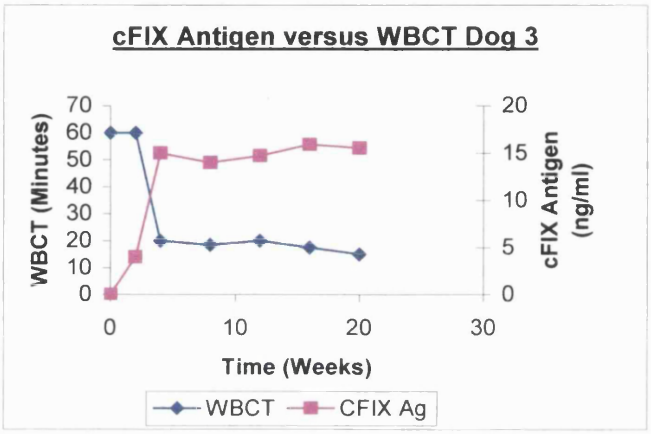


As can be seen at the doses injected both of the dogs failed to reach sufficiently high enough levels to attain the correction to take a haemophiliac from a severe phenotype to a mild category i.e. greater than 1%. The next stage of the study was therefore to proceed to a higher dose.

Dogs 3 and 4

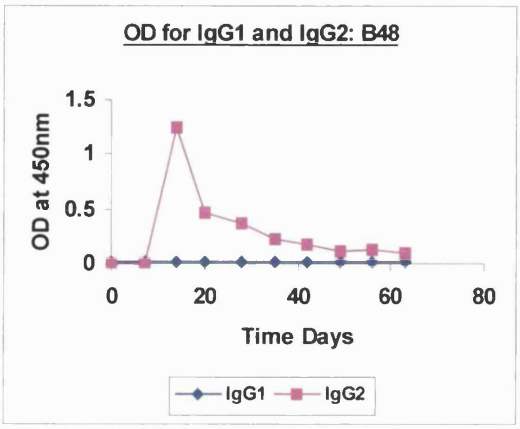
A third dog was then injected at a dose of 3.4 $x10^{12}$ vector genomes per kilogram. Again early on, (< 2 weeks), the whole blood clotting time was partially corrected (15-20 minutes), and was maintained. The graph below illustrates the canine factor IX antigen levels against time

Figure 67 *Canine FIX Antigen Dog 3*



A non-inhibitory antibody was detectable by day 14 as determined by western blot and elisa. The subtype of antibody was classed as an IgG2 subtype *(fig 68)*. This was performed using a polyclonal sheep anti canine IgG that does not cross-react with other immunoglobulins. The exact mechanism of this type of antibody formation has yet to be elucidated but it may reflect a weak B cell response without T cell help.

Figure 68 *Antibody titre dog 3*

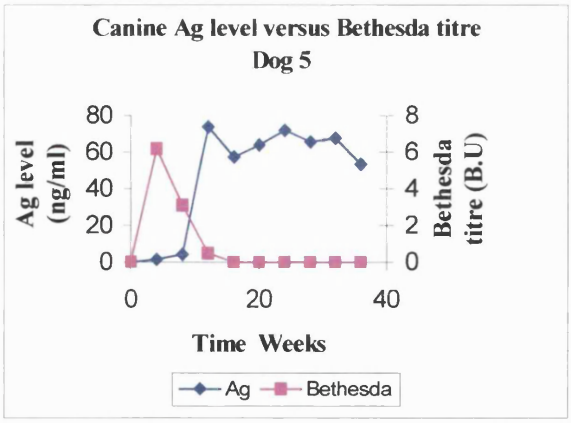


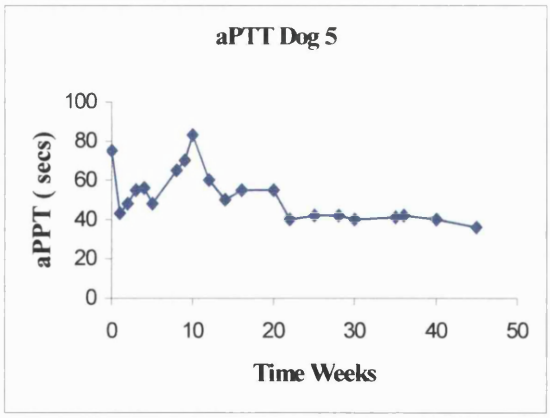
The production of this antibody was self-limiting, but the antigen levels in this animal was too low to reach therapeutic benefit. A fourth dog was injected at a similar dose but again the antigen level attained was too low to be of therapeutic benefit.

Dog 5 Inhibitory antibody

The fifth dog received an injection at a dose of 8.5x 10^{12} vector genomes /kg AAV-CMV- cFIX. The WBCT on day 4 and 7 were corrected, however this increased by day 14 and remained at pre-treatment levels until day 77. An inhibitor screen revealed prolongation of the APTT of normal plasma at days 14, which then markedly prolonged at between days 28-42. Further analysis of this dog, showed that this dog made an inhibitory antibody as determined by Bethesda assay. The appearance of the circulating inhibitor coincided with a fall in the circulating canine factor antigen (*fig 69*) .The graphs below illustrate the fall in antigen level and the appearance of the inhibitor.

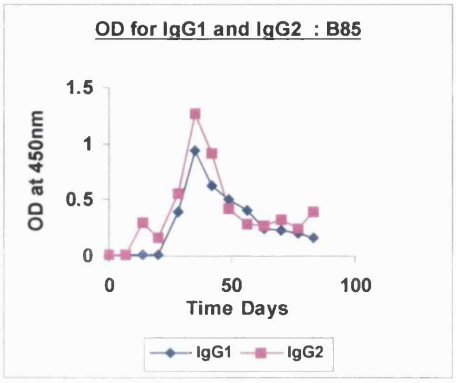
Figure 69 *Canine antigen dog 5*





When analysed further the antibody was inhibitory in nature as seen by the Bethesda titre and the increase in APPT level to baseline. Subclass analysis showed that this dog produced a further subclass of antibody to factor IX i.e. both IgG1 and IgG2. This is illustrated below:

Figure 70 *Antibody data Dog 5*



This graph shows the appearance of both an IgG1 and IgG2 subclass antibody to the canine factor antigen.

The inhibitory antibody response was self-limiting as evidenced by the subsequent reduction in Bethesda titre over time. This occurred with no further therapy to eliminate the antibody i.e. no high dose concentrate infusion. The dog was later subsequently challenged with normal canine plasma (D110-D112) and failed to demonstrate any signs of an amnestic response.

Dog 6 (D31)

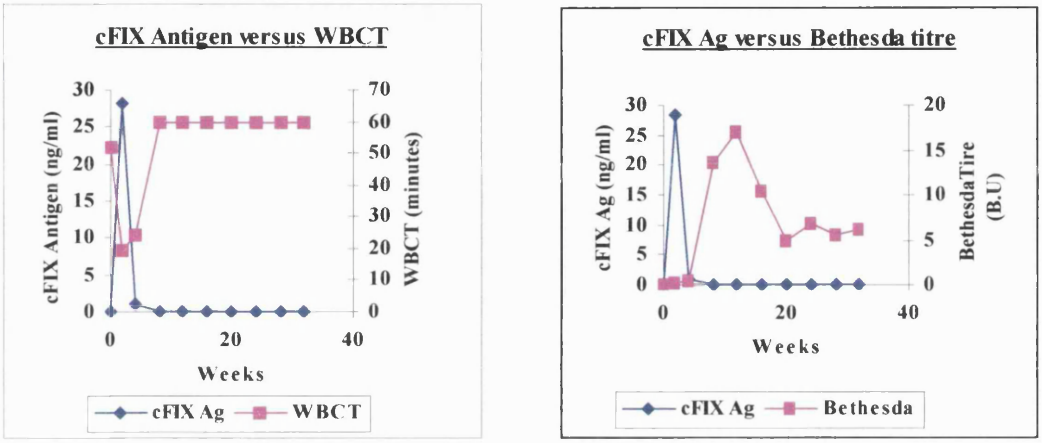
A sixth dog was injected with a similar dose of vector at 8.4 x 10^{12}/kg. This dog also made an antibody, which was transient and non-inhibitory. The WBCT was prolonged D14 –42 and from D49 began to shorten again with a rising factor IX level. In this dog cf. Dog 5, which made an inhibitory response, there was no inhibitory antibody made. The results from dog 5 and 6 suggest that there may be a threshold dose above which antibody formation is induced. It appears though there is no discrimination at this dose as to the nature of the antibody induced i.e. whether inhibitory or not. In order to look at this further, the next dog was injected with an even higher dose of vector.

Dog 7 B14

This dog was injected with a dose of 1.1 x 10^{13} vg/kg, and again early correction of the WBCT was noted to 15 minutes at 2 weeks post injection. However at 4 weeks post injection the WBCT increased and subsequently the dog produced an antibody to

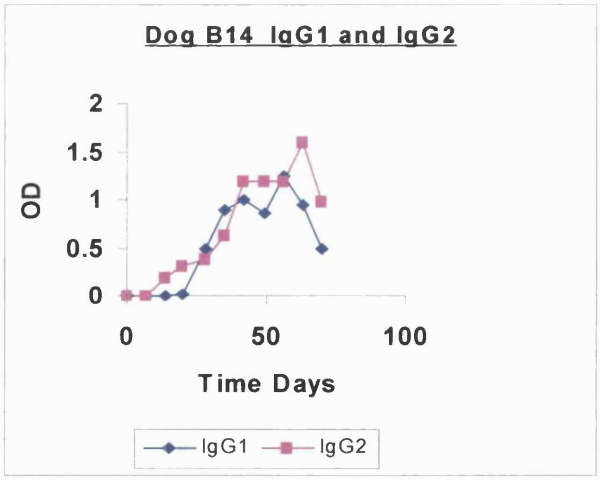
canine factor IX detectable by western blot and Elisa. Additionally as observed in Dog 5, this antibody was inhibitory as evidenced by Bethesda assay (*fig 71*).

Figure 71 *Dog 7 WBCT time and Bethesda titre*



A more detailed analysis of the subtype induction in these cases once again revealed the presence of two subclasses being induced IgG1and IgG2.

Figure 72 *Antibody subclasses dog 7*



The presence of a further subclass of antibody induced may reflect further induction of additional CD4 T helper cell subsets. Because of the previous observations in the fifth, dog (i.e. the occurrence of a transient inhibitory antibody) and in this dog, it was

anticipated that an inhibitor response may be observed. In order to study this in more detail, further characterisation of the observed immune responses was made by looking at the cytokine profiles in the CD4 Th helper cells in this animal. This information would be helpful in determining what type of T helper response cell was occurring. Therefore in this animal, a study of prospective T cell proliferation indices was undertaken at 2 weeks, 8 weeks and 12 weeks post injection and the results of this are outlined later in this chapter.

Conclusions

All of the injected dogs displayed early correction in their WBCT which was sustained at the time of writing for greater than 1 year, indicating persistent expression and biological activity of muscle derived factor IX. However the levels of factor IX expression were low until a certain threshold in dose was reached.

From the results appeared that the presence of an inhibitory antibody was induced once above a threshold of $>8.5 \times 10^{12}$ vector genomes per kilogram. In these animals, it appears that the inhibitory antibodies were associated with both IgG1 and IgG2 subclasses. However, in the transient non-inhibitory antibody cases only an IgG2 subclass was observed. These results suggest the possibility of further CD4 helper subsets induction in the inhibitor dogs. These results show that inhibitor formation may be related to the increasing dose of vector used. i.e. when a threshold dose of $8.5 \times 10/^{12}$ vector genomes/kg is reached.

8.4.1 Cellular Immune responses seen

As described earlier in a gene therapy approach, there is the potential for the protein to be produced intracellularly and be presented to the immune system in an MHC class I pathway. The results obtained from the small animal model showed that there is no damaging CTL response to the transduced muscle in murine haemophilia B. However because of a paucity of the correct homologous reagents in the canine model it was not possible to develop a CTL assay to directly look at this in canine hemophilia B. However histological analysis was performed to look for the presence

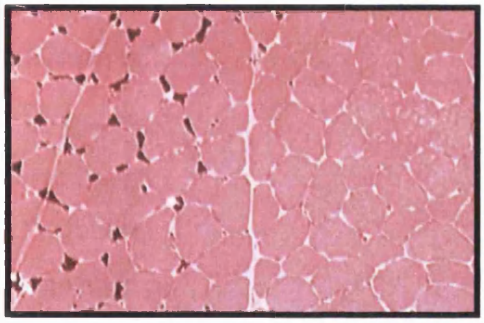
of inflammatory infiltrates by haematoxylin and eosin staining, and for the presence of CD8 infiltration by immunofluorescence.

1) *Histological analysis*

H&E Staining

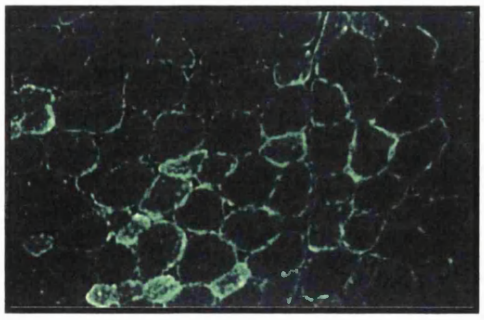
In a preliminary experiment 2.8 x 10^{11} AAV cFIX vector particles was injected into the tibialis anterior muscle of a phenotypically normal dog. Biopsies of the injected tissue were taken from where the carbon particles were localised. Serial sections of the injected muscle revealed no evidence of any inflammatory infiltrated as determined by H&E staining and immunohistochemistry for CD8 analysis

Figure 73 *Histology canine muscle*



The section here shows a normal architecture of the injected muscle. The black particles seen are carbon particles, which are mixed prior to injection with the vector suspension. This aids localisation of the injected muscle when the muscle is excised.

Canine factor IX antigen



In order to look for FIX antigen expression sections were stained serially by immnunohistochemistry to look for the presence of canine factor IX antigen.

Immunohistochemical CD8 staining

This was performed on the injected muscles to look for evidence of CD8 infiltration. In the AAV injected dogs there was no evidence any CD8 infiltration indicating a relative absence of any inflammatory cellular infiltrates.

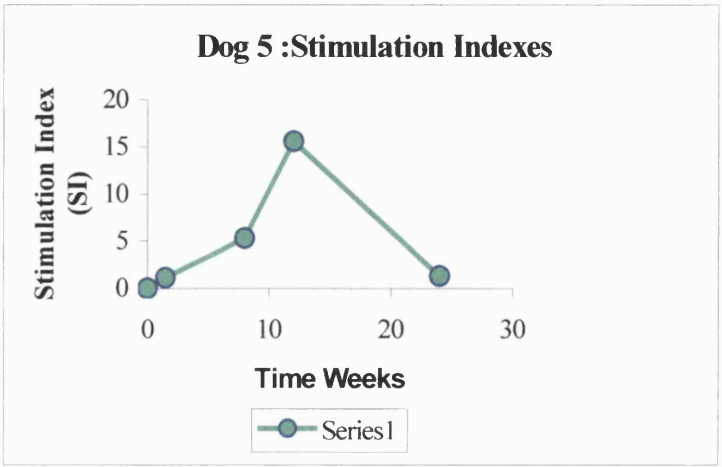
2) *Cellular responses*

The observed antibody responses in dogs 5and 7 suggested a T cell helper response was occurring which induced antibody formation. Therefore, a cellular proliferation assay was set up to look for evidence of cellular proliferation to canine factor IX in the peripheral mononuclear fractions of the injected dogs. I wish to acknowledge the helpful comments by Professor Felsburg at the Veterinary School in the university of Pennsylvania (USA) and Dr Michael Day at the Bristol University (UK) Veterinary School in setting up this assay (Day, 1999).

Results of the Stimulation Indexes

In the fifth dog injected, administration of 1.1 x 10 ^{13} vg/per site resulted in a maximum inhibitor titre of 24.5 BU followed by a slow decline to roughly 1 BU. Proliferation of T cells derived from PBMCs and stimulated *in vitro* with cFIX protein was high during the first three months after inhibitor formation (stimulation index < 15). Low or absent T cell proliferation was observed at later time points without inhibitor formation (*data not shown*).

Figure 74 *Stimulation indices*



The T cell proliferations observed in this animal indicate that a *T cell dependent* immune response was occurring during this inhibitory antibody response.

8.4.2 Canine Cytokine Analysis

Intracellular cytokine estimation of proliferating cells

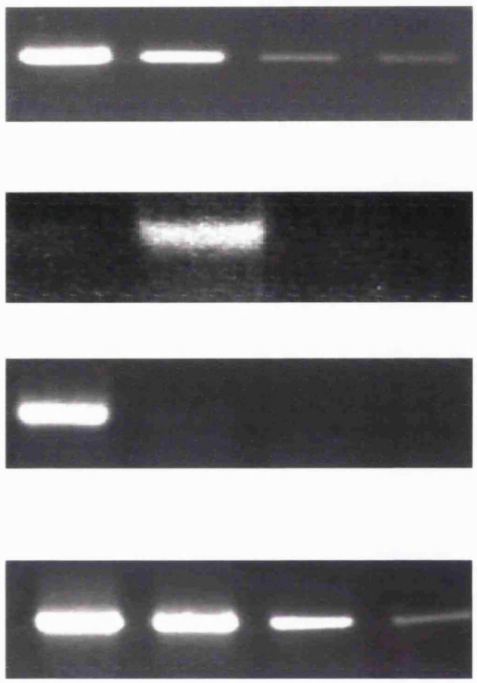
An additional step was incorporated in the protocol to determine the cytokine patterns produced by the proliferating cells. Using a RT PCR technique, first extracting the lymphocyte mRNA, and then performing a RT PCR reaction to look for the presence of intracellular cytokines IL-2, IL-10, IL-4, and Interferon gamma in the proliferating lymphocytes performed this. This modification was primarily performed because of a lack of available canine reagents to measure cytokine levels by Elisa in the proliferating wells by conventional cytokine release assay.

RT PCR Analysis of Intracellular Cytokines

In order to look at the cytokine profiles generated in Dog 7, mononuclear cell cultures were obtained by the method as described above, and plating out the cells at 10^5 per plate, and setting up a proliferation culture to canine factor IX antigen. The cells were assayed at 48 and 72 hours to look for expression of intracellular cytokines by an RT-PCR method. (The method is described in materials and methods in chapter 3, page 81). The proliferating cells were harvested at 96 hours and cellular RNA was then extracted and then an RT PCR assay was performed to generate cDNA for analysis of the four cytokines IL-4, IL-2, IL-10, and Interferon gamma. A mock uninjected dog was used a control, and the internal control amplified gene was β actin. The results are described in the following diagram:

Fig 75 *RT-PCR for intracellular cytokine expression in canine T cells*

Haemophilia B dog:	Dog 7	Dog 7	Dog 7	Uninjected Dog
Invitro Stimulant:	**Con A**	**cFIX**	**Mock**	**cFIX**



The results of the RT PCR reaction are shown above. In the reaction, Concanavalin A was used as a positive control to stimulate the lymphocyte cells and the internal control used was ß actin (a constitutive gene), which shows that RNA was being amplified up in the PCR reaction.

For the test samples with cFIX used as the stimulant, IL-10 was produced from the proliferating cells along with IL-2, however no interferon γ detectable.

The profile described here for canines would indicate both in mice and humans, a Th2 profile in keeping with a humoral driven response. Although the cytokine profiles for the canine species are not completely described, if one extrapolates the results from both murine and human species, the results also appear to be in keeping with Th2 cellular driven response.

8.5 Immunodulation Strategies to Overcome Inhibitory Responses

As as result of the observed responses in dogs 5 and 7 (i.e. the development of inhibitory antibodies), strategies were devised to try and avert inhibitor formation. Based on the experiments using haemophilia B mice that are at high risk for inhibitor formation because of a large factor IX (FIX) gene deletion, a protocol was developed that was aimed at avoiding inhibitor formation following intramuscular (IM) administration of an adeno-associated virus AAV vector expressing FIX.

Aim

The aim of the experiment was to try and avert inhibitor formation by the use of the immunmodulator drug, cyclophosphamide. A total of four doses of cyclophosphamide was given around the time of vector administration starting from day 0 and then biweekly thereafter upto week 6. This treatment efficiently prevented antibody formation against murine FIX in the mouse studies.

Method

The dogs used in this study (Dogs 6 and 8) were littermates and went onto receive similar doses of vector but the second dog (dog 8) additionally received cyclophosphamide to try and avert inhibitor formation. Dog 6 received AAVcFIX vector at a dose of $8.5x10^{12}$ vector genomes in 16 sites via intramuscular injection. This dog did not receive any further immunosuppressive treatment .Dog 8 received a similar dose of vector ($8.5x10^{12}$) but additionally received a total of six intravenous infusions of cyclophosphamide (*200 $\mu g/m^2$*) at the day of vector administration and during weeks 2, 3, 4, 5, and 6.The dosing schedule is outlined in the table below:

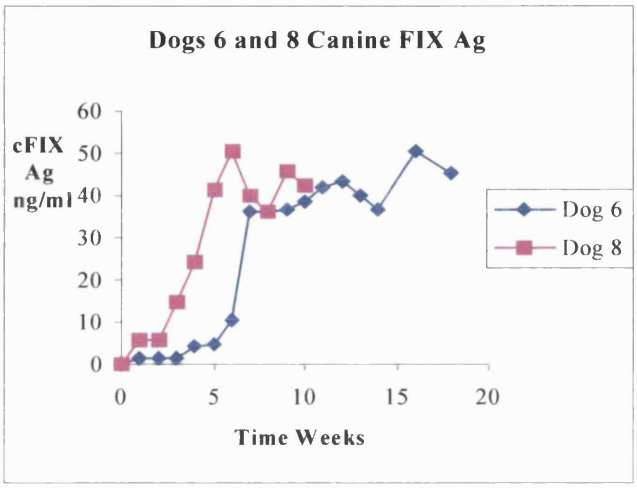
Table 12 *Dosing Schedule immunomodulation in dog 6&8*

Variable	Dog 6	Dog 8
Cyclophosphamide	No	Yes
Vector dose per IM site	10^{12} vector genomes	10^{12} vector genomes
Number of IM sites	16	13
Vector dose per kg	$8.5x10^{12}$ vector genomes	$5.6x10^{12}$ vector genomes

Results

The figure below shows the cFIX Ag expression attained in both dogs.

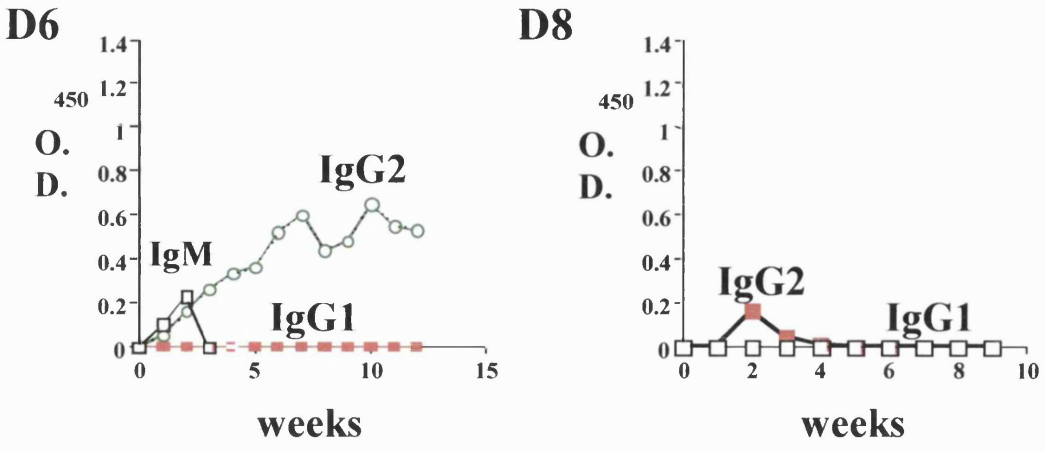
Figure 76 *Canine FIX Antigen Dogs 6 & 8*



The antigen results show that initially Dog 6 did not show expression of antigen. There was the appearance of an antibody in the plasma of initially IgM antibody followed by IgG2 antibody. In the dog treated with cyclophosphamide (Dog8) there were minimal levels of antibody detectable both by Elisa and western blot as shown below.

Figure 77 *Elisa Antibody titre Dogs 6 & 8*

1) Elisa of Dogs 6 and 8 looking for IgM, IgG1, IgG2



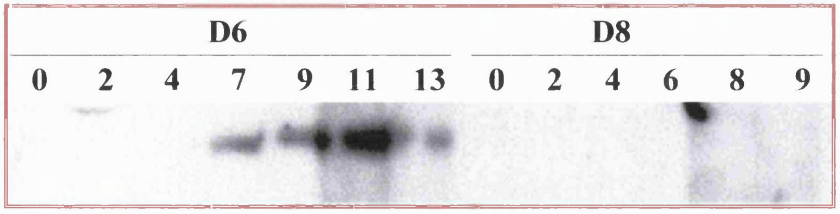
As can be seen from the above diagram Dog 6 continued to make detectable amounts of the IgG2 subclass. However in comparison the IgG2 made by Dog 8 was suppressed.

2) Western Blot

Method

Western blot analysis for detection of anti-cFIX in canine serum samples is shown below. Briefly, Canine FIX (1 μg/lane) was transferred onto a nitrocellulose membrane and incubated with canine serum samples (1:200 dilution). Antibody-cFIX complexes were detected with anti-canine IgG coupled to horseradish peroxidase and visualised by ECL (Amersham) detection. Samples were drawn at weeks 0, 2, 4, 7, 9, 11, and 13 for D6 and at weeks 0, 4, 6, 8, and 9 after vector administration for D8.

Figure 78 *Western blot*



The western blot shows that in dog 8, there was no evidence of antibody detectable at all the time points measured, however in dog 6 antibody was detectable at weeks 7 to 13.Both sets of the above data (western blot and Elisa) indicate that in the Dog 8 it was possible to block the production of antibodies by the administration of Cyclophosphamide. This result suggests that it may be possible to suppress antibody formation in the setting of a large animal model of haemophilia although to confirm this result more numbers will be required.

8.6 Discussion

In these canine studies, the proof of principle concept of a gene therapy approach in a large animal model for haemophilia was shown; that is to say long term gene expression is possible, with long-term expression of factor IX. These studies represent a significant advance over those carried out by Kay et al (Kay *et al.*, 1994) where sustained levels of factor IX were demonstrated (using a retrovirus), but at levels too low to be therapeutic. The studies presented here indicated that using a different vector and target tissue, up to 20 to 30 fold higher levels were achieved which in one case resulted in a therapeutic sustained level of greater than 50ng/ml being achieved.

The approach taken represents a new strategy for the treatment of haemophilia. This raises the question of inhibitor development against the transgene product canine factor IX. ELISA, western blot and Bethesda assay monitored all dogs for the presence of antibodies. The results described, indicate that these dogs may make an inhibitory response to factor IX when challenged with a vector dose above $8.5\ x10^{12}$ vector genomes per kilogram. A more detailed analysis of the immune responses against canine factor IX suggests an increased likelihood of inhibitor development with an increase of vector *dose per site*. Animals injected with 1.4×10^{12} / site had no detectable anti cFIX (Dogs 1&2) or a highly transient (2 weeks) non-inhibitory antibody, or a transient low titre (2 BU) inhibitor at a later time point (1 Dog) after trauma and extensive infusion of normal plasma. However two dogs injected with $2x10^{12}$ vg/site had either a transient inhibitor (Dog 4) or a transient non-inhibitory antibody, and when this dose was increased further as in dog 7 an inhibitory antibody response was seen. More study of predictable risk factors in a gene-based approach is required, so that this response may be averted. It would be interesting to look at how cytokine profiles differ with escalation of dose.

The inhibitor formation observed, was shown to be T cell dependent as judged by the mononuclear proliferation assays and also appeared dose dependent. The antibody responses appeared to be transient and self-limiting and characterised by spontaneous remission. In the characterisation of immune responses studied, in this large animal model, it is important to bear in mind the mutation under study. In this model, the haemophilic phenotype was caused by a point mutation resulting in no circulating

antigen being detectable, but the presence of mRNA transcripts are detectable. Thus it is possible that canine factor IX protein is produced intracellularly, but destroyed at this stage because of instability of the protein. It may still be possible therefore to present the relative dominant epitopes to the developing immune system so that later antigenic challenge may lead to tolerance. This may explain lower incidence of inhibitor formation seen in the canine model (2/7), as compared to murine of haemophilia (described in chapter 7) where the mutation is caused by a large deletion.

As shown in dog 7, T cell proliferation was observed to canine factor IX antigen indicating the T cell dependent nature of the response. To avert this, ways of blocking the inhibitor response were explored. It was shown that using cyclophosphamide in dog 8, that this agent may be useful in suppressing the inhibitory response. More numbers will be required to confirm this result. Other methods aimed at inducing tolerance to the transgene product should be explored such as blocking of co stimulatory responses with immunomodulatory agents such as CD40 ligand, and CTLA-4 Ig. The difficulty presented here is the requirement to have the relevant species-specific homologous reagents available for study in the canine model. Equally important, will be the testing out of further mutation types in large animal models, and their likely effect on the development of inhibitor formation. In any new developmental therapy for haemophilia, it is important to look prospectively for the development of inhibitors.

The studies presented here, show unequivocally that a gene therapy approach in large animal appears safe and efficacious and provides the rationale and pre clinical data to support translation of this approach into studies involving human subjects.

CHAPTER 9

SUMMARY OF RESULTS

The results produced in this thesis may be divided as they relate to the relative components of the immune response as described in the *Figure 11*, page59.

The results identified 6 major areas, which appear to characterise and influence the occurrence of an immune response to a gene transfer process. These are as follows:

- a) The role of the vector in a gene based approach
- b) The role of the transgene used in a gene based approach
- c) The role of the animal model
- d) The mechanism of antibody formation in a gene based approach
- e) The occurrence of whether a cellular CTL response was induced.
- f) The role of the underlying mutation in the recipient animals of gene transfer

The results are depicted in tabular form on the following page.

The initial experiments in chapter 4,5 look at the isotype profiles induced via different vector selection. The results demonstrated that the response was a T cell dependent process. The likelihood of an immune response was also correlated with the underlying mutation present (Chapter 7,8). The profiles generated were in keeping the results of the CTL experiments, which demonstrated that the presence of such a response correlated with a dominant IgG2 profile (Chapter 6).

Other exogenous factors such as the role of the animal model and route of gene delivery were also studied and clearly shown to influence the outcome of an immune response (Chapters 5,7,8). Finally because of the occurrence of an immune response, immunomodulatory strategies were sought to overcome the process, and this formed the final facet studied (Chapters 7,8).

Table 13 *Results summary*

Facet Studied	Specific Result(s)	Evidence
a) Role of the Vector	The nature of vector selection Influences response to the transgene product	Differing antibody profile Adenovirus : IgG2 Adeno associated virus : IgG1 Plasmid: IgG2
b) Role of the transgene	Non Homologous transgenes generate immune responses	Injection of AAVhFIX to Ab production Injection of AAVmFIX fails to elicit Ab responses
c) Role of the Animal model	C57BL6 strain produce high circulating factor IX Different strains do not result in high circulating factor IX	Serum estimation Balb/c , C3H strains
d) Mechanism of Antibody formation	Is a ***T cell*** **dependent**process	Antibody isotype profiles in mice Cellular Proliferation data in mice, dogs
e) Presence of a CTL response to a secretable transgene	AAV mediated **-ve** Adenovirus **+ve**	***In vitro*** CTL , ***In vivo*** CTL Antibody profiles
f) Role of the mutation	Correlates with inhibitory outcome	Injection AAVmFIX to **gene deleted** mice results in Ab production in all animals Injection AAVcFIX to ***mis*** **sense** mutation results in Ab production 1/5 animals
g) Immunomodulatory strategies in Antibody formation	It is possible to avert antibody formation in a gene based approach	Cyclophosphamide, FK5O6 CD40L Ab

CHAPTER 10

DISCUSSION

Evolution of Therapies

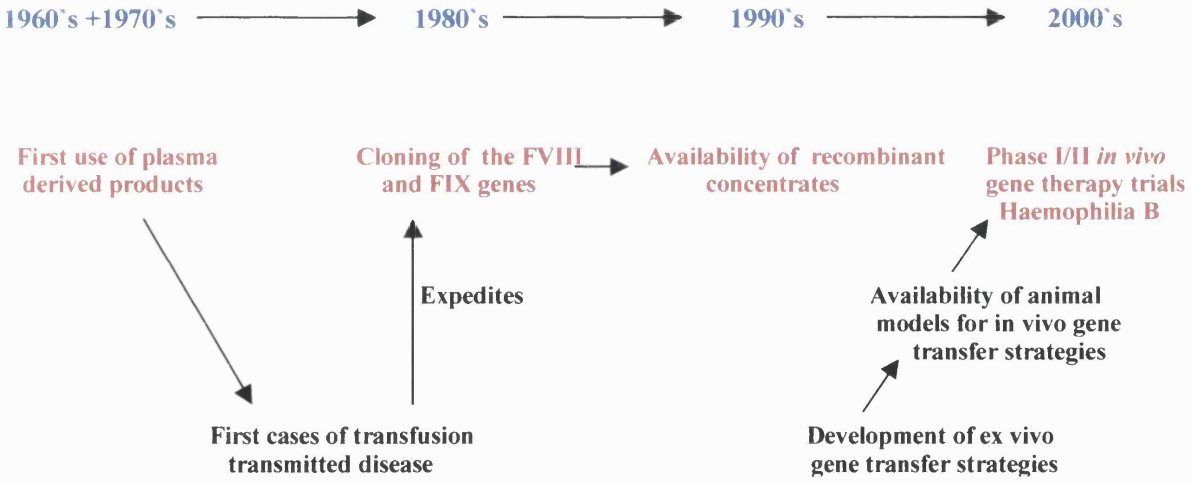
The desire to cure a genetic disease by a gene therapy has been and is the dream of both scientists and haemophilia treaters alike. Haemophilia is an example of a disease that has undergone a dramatic revolution in treatment of the last 50 years. From being a disease with a high mortality, it is now a disease where now it can be expected that most patients can live relatively trouble free lives. These treatments have come about as the result of the recognition that the disease related complications could be prevented and ameliorated by the infusion of clotting factor concentrates and maintaining a clotting factor above 1%.

Despite these advances, haemophilia is an example of a disease where the new treatments did provide the necessary efficacy, but at a cost. The unknown contamination of plasma derived clotting factor concentrates resulted in devastating consequences for the haemophilia population with many patients becoming infected with hepatitis viruses and Human immunodeficiency virus. This led to a drive to produce purer clotting factor concentrates by recombinant technology circumventing the need for plasma-derived concentrates and their inherent associated risks. The result was the production of "state of the art" factor VIII and FIX recombinant concentrates for treating haemophilia A and Haemophilia B. Despite this, the high cost of these products makes their use prohibitive in several areas of the developing world and also in parts of the western world, including the United States where routine prophylaxis is not freely available.

In the developing world there are virtually no countries using these products unless in special circumstances. This means that many patients in these countries still do not receive treatment for their diseases resulting in chronic morbidity and mortality. Many babies born with haemophilia in African countries still die as a result of bleeding complications of circumcision, often, which go unreported (*Personal communication to me by a father of a little boy in Kenya with haemophilia*). The use of a simple easy administered treatment in these countries would ameliorate the suffering of many untreated children.

The next technological stage of progression from the use recombinant products would be the *in vivo* production of normal clotting factor in a human from a gene based approach Thus the need for a gene based approach is clear to see, and offers the only real hope for cure of this disease. This realisation would represent the natural progression of the advances made both in the understanding of the early descriptions of the disease in the Talmud right through to the molecular advances of the twentieth and twenty first centuries. It would represent a true evolution of therapies for this disease.

Figure 79 *Evolution of therapy for Haemophilia*



As can be seen from the diagram above, several of the new therapies have resulted in potentially damaging complications i.e. transfusion transmitted disease from plasma derived concentrates. The dawn of a gene-based approach for haemophilia also brings with it the possibility of complications as with any novel treatment for haemophilia. As described earlier a gene based approach brings with it hope, but also the potential for treatment related complications and at present limitations to the overall efficacy of the process. These limitations at present centre around vector design and the issues of

incorporating genes into them. This is exemplified by AAV where the maximal insert capacity is 4.9 Kb is sufficient for FIXcDNA, but not for FVIII.

Other inherent problems related to vectors include engineering ones with high transduction efficiency so optimal gene expression may be obtained. The results of the current ongoing muscle directed trial demonstrate that although gene expression is observed in the injected tissue, the circulating clotting factor levels obtained are too low to be therapeutic indicating insufficient gene expression of the target tissue. Finally, other issues such, as the amount of vector that can be produced safely under GMP facilities is a rate-limiting step for many current gene transfer protocols.

Immune Response Complications

The other major complication of a gene-based approach is the occurrence of an immune response. This complication, like any other conventional treatment for haemophilia is a distinct possibility, and one that requires a critical analysis before the treatment can be deemed safe enough to be offered as a routine treatment. The aim of my studies was to examine some of these issues surrounding a gene-based approach. At the outset of this thesis, gene therapy was still very much in its infancy and simple preconceptions about pre clinical gene transfer were often simplistic, misunderstood and misguided.

An example of this, were the numerous studies carried using non species-specific trasnsgenes, making results often difficult to interpret when extrapolated to human studies. This partly stemmed from the lack of availability of species-specific homologous transgenes for study. This also hampered the development (by gene targeting technology) of the relevant animal models for study. Fortunately, the development of the relevant animal transgenes has allowed for the acceleration of studies to be carried out appropriately so that immunological studies may be specifically designed to answer a particular question and allow predictability.

Role of Vector in gene transfer

The studies in chapter five examined the role of the vector in gene transfer, and showed that this was particularly important in the immune responses generated. The results showed that both plasmid and adenoviral vector transduction resulted in more dominant IgG2 profile in mice which correlates with a Th1 cellular antibody dependent response. Although this gave a clue to the type of immune response occurring i.e. by interpretation of the humoral profile, a more definitive approach to answer whether also a cellular cytotoxic response was occurring would be to set up a CTL assay to look at this. This complex assay took many months to develop. Importantly using this assay it has been possible to show that Adenoviral based transduction in muscle resulted in strong cytotoxic T cell responses to the transgene product human factor IX in both normal and haemophilia B mice (chapter 6). Similar experiments were performed using an Adeno associated virus, which failed to elicit CTL responses when encoding the human transgene factor IX in a muscle directed approach. This was a particularly important result, since most of the previously documented experiments using an AAV vector were performed a non-secretable transgene such as the ß galactosidase reporter gene (Yang *et al.*, 1994b; Yang *et al.*, 1995c; Fisher *et al.*, 1997; Xiao *et al.*, 1997). The result confirmed earlier findings from the antibody isotype data, which predicted a lack of a CTL response i.e. an IgG1 dominant profile. The results of the *in vitro* CTL experiments were later followed with an ambitious *in vivo* CTL experiment in which adoptively transferred allo reactive lymphocytes failed to induce an *in vivo* CTL response (Fields *et al.*, 2000).

Role of the Transgene in gene transfer

A critical feature to any gene based protocol is the rationale design of the transgene which when translated intracellularly will lead to effective production of the desired protein product with the relative absence of an immune response to the transgene product. Early experiments performed in gene-based protocols often incorporated the use of reporter genes to demonstrate efficacy of the procedure. Sometimes these same reporter genes were also studied *in vivo* and not surprisingly lead to the development of humoral and sometimes cellular immune responses. The early experiments in chapter 5 showed that the use of a non-homologous human transgene in mice lead to

the development a humoral response to human factor IX. When further similar experiments were performed with a murine transgene no immune responses were observed. This may seem on the surface a predictable result, but at the outset of these studies, many publications presented data with the use of non-homologous transgenes such as the reporter gene β galactosidase(Yang *et al.*, 1994b; Yang & Wilson, 1995; Olthoff *et al.*, 1997). Clearly, these results can never be extrapolated to using appropriate homologous transgenes in an optimal system. The results in chapter 5 demonstrated the crucial importance of incorporating the relevant homologous transgenes into vectors in order to accurately assess immune responses.

Strain variation

The results of chapter 5 also examined the role of other components influencing the immune response to gene transfer and particularly the route of administration in different strains of mice. The results of these experiments concluded that the haplotype of a mouse may be an important determinant of the immune responses observed. In particular the C57BL6 strain of mouse seemed to be permissive to high dose factor expression when transduced intravenously by Adenovirus expressing a human factor transgene. This would seem a highly unexpected result since firstly Adenoviruses are known to highly immunogenic, and secondly the transgene selected was non-homologous.

The mechanisms underlying results were explored and it was found the absence of the class II alleles IE was not responsible for this result. Similarly, it was also found that the immunosuppressive cytokine IL-10 was not responsible for these observed effects. These results point out that what happens in one strain of a species may not representative of other strains in the same species (Fields *et al.*, 2001a). This is important, since many of the early pre clinical studies performed in mice were studied in the C57BL6 strain and may therefore give misleading results i.e. this particular strain of mice may be particularly permissive to gene transfer when challenged with known immunogens.

Role of Mutation / Genotype

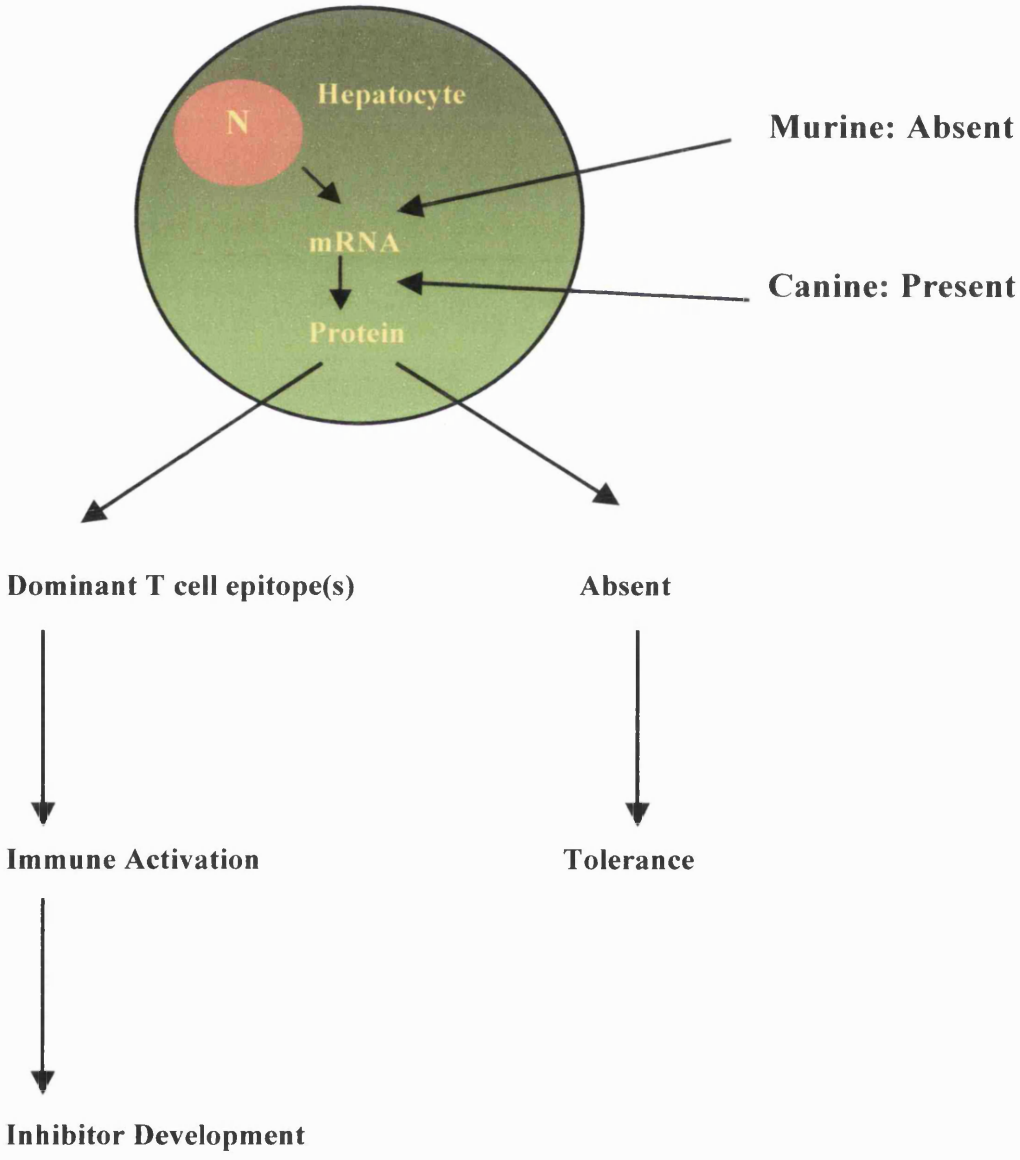
It is known that the immune response may differ from species to species, and a response observed in one species may not be necessarily representative of what may occur in another species. Part of any pre clinical testing strategy for the efficacy of a gene transfer approach is to test out the protocol in a large animal model of the disease. Haemophilia is fortunate in that there are canine models of both haemophilia A and B. Chapter 8 examined the role of gene transfer and the immune responses observed in a canine model of haemophilia B.

The canine model studied was different to the murine model, in that the mutation giving rise to the severe phenotype was caused a *mis*-sense mutation resulting in the production of FIX mRNA which is detectable by a PCR assay (Evans *et al.*, 1989). No circulating FIX protein is found, since the protein-produced intracellulary is unstable, and destroyed before it is able to be secreted. The result is that some FIX peptides may be produced and allow thymic education and tolerance to some of the molecules immunodominant epitopes. This is a different scenario from the murine model, where the phenotype is cause by a large gene deletion resulting in no mRNA transcripts and protein being produced (Lin *et al.*, 1997). The murine model therefore represents the worse case scenario for inhibitor generation as compared to the canine model.

The results observed from the canine experiments produced a transient inhibitory antibody in one animal, which was self-limiting and did not produce an amnestic response when challenged with infusion of plasma. The antibodies produced in the dogs were T cell dependent as evidenced by the results of the proliferation assays. These results are in keeping with current treatment of haemophilia by infusion of clotting factor concentrates. Not all the dogs produced inhibitory antibodies (4/5), and only the dog that received the highest dose made an inhibitory antibody. It will important to reproduce these results, and test out the influence of vector dose and vector dose per site to learn more about the pathogenesis of inhibitor formation in this setting. Contrarily, all of the haemophilia B mice that carry a large gene deletion produced inhibitory antibodies within 8 weeks of vector administration. The results of these experiments point out the significance of the role of the underlying mutation

present, since it appears that the those animals with a more severe defect resulting in loss of coding material are at increased likelihood of inhibitor development. This echoes protein-based situation, where induction of inhibitor formation appears to correlate with severity of the mutation leading to a severe haemophilic phenotype. It will therefore be important in any gene-based protocol in humans to define the underlying mutation present prior to embarking with a gene-based protocol. The influence of underlying mutation is shown in the figure below:

Figure 80 *Relationship between mutation and inhibitor development*



Strategy of immunomodulation

Because of the observed responses occurred i.e. formation of inhibitory antibodies in both animal models of gene transfer, it is important to explore strategies to circumvent this. In the conventional protein based approach, suppression of inhibitor formation is very difficult to overcome once it has occurred. Immune tolerance regimens have been employed (sometimes with use of concurrent immuno suppression) to overcome the response. This is very costly and does not always succeed. The overriding feature of these approaches is to overcome the antibody responses to the circulating protein. The use of high dose concentrates +/- immunosuppression is designed to clonally delete or exhaust those alloreactive T cells involved in inhibitor formation.

Another way to eliminate these cells would be to effectively anergise them at the point of contact with immune inducing epitopes. This may be accomplished by interfering with co stimulatory pathways involved in T cell activation, i.e. blocking of signal 2 i.e. B7.1/7.2 with CD28 on potential cognate CD4 cells, interaction of CD40L/ CD40, interaction CTLA4 with CD28. In the murine animal model of haemophilia, co stimulatory blockade was attempted with the use of drugs and selective antibodies. Some limited success was achieved with use of CD40L antibody, although this was short lived. Similarly, administration of the agent FK506 produced a short-lived response. Use of the drug cyclophosphamide managed to block inhibitory antibody formation in three animals, and produced attenuated responses in others. This agent probably works by clonally deleting the alloreactive clones involved in inhibitor formation. The results obtained were not always predictable, and more sophisticated ways of blocking inhibitory responses are required. An important overriding principle of these results suggests that it is possible to block inhibitor formation given at or around the time of vector administration. To do this, more effectively further knowledge of the biological pathways involved in the immune pathogenesis of inhibitor responses will be required both in the protein based and gene-based approach.

Implications of the work for gene therapy

Any gene transfer approach needs to be safe and efficacious. For a gene therapy approach to be successful, preclinical studies are required to show proof of principle. This needs to be shown first *in vitro* and then in both small and large animal models of the disease. Once these criteria are met, it would seem feasible to progress to clinical trials. The implications of the work presented in this thesis suggest that the immune response observed to a gene transfer approach can be influenced by many different factors. Crucially vector selection seems to strongly influence the response, and cause the formation of both humoral and cellular responses to the transgene product. The transgene itself should also be homologous to the animal it is being tested in to allow an accurate assessment of the immune responses occurring. Host cell factors need to be taken into account such as the nature of the underlying mutation; which should be known prior to any protocol being instituted. The results of the immunmodulation work suggest that this strategy may be successfully employed in overcoming the immune response, and this may be of further use in the future, when it may be needed to be combined with a gene based approach to make the whole process work.

Future work

The results produced from this work showed that a gene-based protocol in muscle using AAV directed gene transfer failed to elicit a CTL response. However, humoral responses were observed which appeared to be dose related in that a certain threshold dose triggered off inhibitory antibody formation. Suppression of these responses was possible with use of immunomodulation given around the time of vector injection. The results produced by the large animal canine model and the ongoing human clinical trial show that the muscle directed approach appears safe, although the levels of gene expression and clotting factors levels attained were low, and not sufficient as yet at this time to translate into therapeutic efficacy (Kay *et al.*, 2000). In order to achieve therapeutic levels, gene expression needs to be higher and any inhibitory antibody response needs to be blocked should it occur. In order to achieve higher gene expression, injecting more sites with the vector could do this. However, this is not practical, since it may involve many more sites and at least 60-70 injection sites,

which is not clinically feasible. Another way to produce more expression is try and gather information/knowledge as to what the biological factors governing AAV based transduction in muscle are. Ways of achieving higher expression have been explored and include using different serotypes of AAV vectors. The serotype selected for use in these experiments was AAV-2. Preliminary (unpublished) work in the laboratory suggests that serotype 1 vectors give x 20-40 increased gene expression when compared to serotype 2 (Arruda *et al.,* 2001). This would increase gene expression but would bring an increased risk of inhibitor formation because of the expected increase in circulating clotting factor expression. Therefore, this strategy would require immunomodulation to suppress these responses.

Another potential way of ensuring higher doses is to increase targeting to muscle by a better delivery system than simple injection. Currently ways are being explored to achieve this, by selecting vascular arterial routes, which supply the target muscles involved. So-called "isolated limb perfusion studies" are under way to explore this approach. Preliminary results suggest that approach may yield much higher levels of clotting factor protein.

Finally, a third approach would be to select an alternative target tissue, which may yield higher gene expression levels .An obvious choice is the liver, since it is the known site clotting factor synthesis normally. Published data suggests that the levels attained by this approach yield higher levels of clotting factor, but there are fears using this route about the possible increased risk of inadvertent germ line transmission. Currently studies are ongoing to define these risks.

Other future work should centre around the biology of the immune response, and which specific cell types are involved .In particular the cell types in each target tissue involved in antigen presentation may give further clues to local and systemic immune response.

Summary

It is now approximately 10 years since the first papers appeared showing that it was possible to transfect several different target tissues and produce biologically active clotting factor proteins. The concept of a gene-based approach is easy to realise but poses several difficulties in practice. These were hampered at the beginning of the previous decade by the lack of available tools to study the process preclinically before moving to clinical trials. The cloning of the relevant homologous transgenes and simultaneous development of their cognate animal models has facilitated rapid advances in demonstrating that the proof of principle works in an *in vivo* setting.

We have now moved from these preclinical studies into three ongoing human clinical trials. One of these studies using AAV in a muscle directed approach is nearing completion, and has proceeded uneventfully with no severe toxicity being demonstrated (Kay *et al.*, 2000). However, the levels of gene expression remain too low to yield satisfactory therapeutic circulating human FIX, but if the process could be optimised with better levels of gene transfer, the prospect of success moves ever closer. The degree of investment being made from the scientific, academic, and corporate sector is enormous and serves to underpin the future success of the "dream" becoming a reality.

Although much still remains to worked out, one thing is certain, the progress made from a decade ago is vast, and it remains only a matter of time before haemophilia becomes one of the first human genetic diseases to be cured by this approach. The ability to arrive at this position would be a great feat for mankind and be the culmination of many decades of scientific and technological advances. Equally important it will bring hope to millions of sufferers afflicted with other human diseases, which may also, one-day benefit from these advances.

Postscript to the work

Since the completion of my work, the results of the preclinical data provided sufficient optimism to lead Professors High`s group to proceed to a Phase I clinical trial using a muscle directed gene based approach in human subjects. The preliminary

data from the trial published in Nature genetics shows has proceeded uneventfully with no severe toxicity being demonstrated (Kay et al., 2000). Importantly no deleterious immune responses have been observed, and are in keeping with that predicted from the pre clinical data. Other trials have also been initiated, including a liver directed approach and the results of these are eagerly awaited.

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